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SCREENING AMONG MICRO AND MACROMYCETES FOR LACCASE PRODUCTION

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Abstract

Laccases show multiple biotechnological application and different fungal groups have been widely reported as laccase producers. The main aim of our research it was to perform on-plate screening for laccase production among different macro and micromycetes, while optimising the screening protocol in the presence of different guaiacol concentrations. From our collection were taken into account filamentous fungi belonging to the following species: Aspergillus clavatus, A. aculeatus, Botrytis cinerea, Neurospora crassa, Trichoderma sp., Penicillium digitatum. Among the macromycetes were tested Laetiporus sulphureus, Ganoderma lucidum, Agaricus bisporus and Pleurotus ostreatus. The screening was performed on PDA added with guaiacol. The positive microorganisms belong to two strains of Trichoderma spp. isolated from soils, one variety of P. ostreatus and two of A. bisporus originating from supermarket wastes. In the case of P. ostreatus, the use of guaiacol higher than 0.1% has inhibited the fungal growth, as well as the halo-formation. In the case of Trichoderma spp. strains the use of guaiacol above 0.1% and till 1% dind't lead to any halo formation, while the mycelial growth was not inhibited; relevant halos were registered for the concentrations of 0.01% and 0.025%, when the maximum was reached after 6-7 cultivation days.

Key words*: fungi, laccase, guaiacol, on-plate screening optimisation.*

INTRODUCTION

In the past decade, enzymes like laccases, have gained great importance for their biotechnological applications. Laccases play an important role in bioremediation and biodegradation, dye decolorization, paper and pulp industry, as well as in the food industry (Couto et al., 2006; Burlacu et al., 2018). Laccases belongs to the enzyme family of copper-containing oxidases catalysing a variety of oxidations and having a broad substrate specificity.

Fungi, macro and micromycetes, have been widely reported as laccase producers. Different Ascomycetes and Basidiomyceste species, like *Lentinus edodes*, *Coprinus comatus, Oxyporus obducens, Fomes fomentarius*, *Ganoderma lucidum*, *Fomitopsis pinicola, Flamulina velutipes, Pleurotus eryngii*, *Pleurotus ostreatus, Agaricus bisporus* or *Trametes versicolor* were reported to have potential for laccase production (Popa et. al., 2018; Albu Proca et al., 2019). Among the filamentous fungi, several species were reported to produce laccase, respectively *Aspergillus nidulans, Botrytis cinerea, Melanocarpus albomyces, Chalara paradoxa, Chetomium thermophilum, Magnaporthe grisea, Podospora anserina, Neurospora crassa, Rhizoctonia solani* or *Trichoderma harzianum* (Albu Proca et al., 2019). Yet, among the fungi group, the laccase production studies are far to be completed and there is still room to optimise the screening methodology and the enzyme production.

Different methods for on-plate lacasse production screening have been reported by now by adding different substances or colour indicators in the media, like ABTS [2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] (Sodent et al., 2002), bromphenol blue (Tekere et al., 2001) or guaiacol (Kiiskinen et al., 2004; Lopez et al., 2006).

Guaiacol is a phenolic natural product first isolated from Guaiac resin and the oxidation of lignin. Nowadays is commonly derived from guaiacum or wood creosote as yellowish aromatic oil. From a biochemical point of view guaiacol is a monomethoxybenzene that consists of phenol with a methoxy substituent at the ortho position (ChEBI database). When screening the production of laccases for bioremediation purposes of various xenobiotics, analyses revealed that guaiacol is much better associated with the decolorization of multiple structurally different dyes (Wong et al., 2013). In this respect, when approaching laccase production for bioremediation application the use of guaiacol may be a successful solution. The reported data are making reference to the use of different guaiacol concentration in the screening for laccase production, and the employed amounts are quite different, starting from 0.01% (Kiiskinen et al., 2004) to increased concentration (1%) for selecting high tolerance laccase producing strains (Devasia & Nair, 2016).

The main aim of our research was to perform on-plate screening for laccase production among different macro and micromycetes, while optimising the screening protocol in the presence of different guaiacol concentrations.

MATERIALS AND METHODS

Microorganisms

In the screening were used different macro and micromycetes isolated and conserved in the microbial collection of the Faculty of Biotechnology from USAMV Bucharest or procured from the market as wastes (Table 1).

Table 1. Mycetes used for laccase's production on-plate screening

Mycetes group	Species/Strain/ Variety	Origin
Micromycetes	*Aspergillus clavatus*	Grapes
	Aspergillus aculeatus	Grapes
	Botrytis cinerea	Grapes
	Neurospora crassa	Cacao beans
	Trichoderma spp. MI2	Soil
	Trichoderma spp. CP	Soil
	Penicillum digitatum	Soil
Macromycetes	*Laetiporus sulphureus* DD	Forest tree from Danube Delta
	Laetiporus sulphureus B	Urban tree Bucharest
	Ganoderma lucidum	Forest tree from Danube Delta
	Agaricus bisporus white variety	Supermarket waste
	Agaricus bisporus brown variety	Supermarket waste
	Pleurotus ostreatus	Supermarket waste

Media

For the inoculum preparation, as well as for the on-plate screening was used a fungal basal medium, respectively PDA (Potato Dextrose Agar). For the visualisation of the laccase production, the PDA was added with guaiacol, as described below.

On-plate screening method for laccase production

For the initial screening of laccase production was employed PDA supplemented with 0.04% guaiacol (Roth Werke GmbH), according to Kalra et al. (2013). Guaiacol was added to the media before autoclaving. The positive test is indicated by the formation of a brown-reddish halo around the fungal culture. In the case of the filamentous fungi (micromycetes), the strains were cultivated on PDA and spore suspensions (10^6 spores/ml) were prepared as inoculum; 100 µl spore suspension was inoculated in the centre of the PDA + guaiacol plate. In the case of the macromycetes, a fragment of 0.5 cm^2 from the mushroom's cap was added on PDA after a partial sterilisation in 70% ethanol. The fungi were cultivated at 28ºC during 8 to 14 days (depending on how fast the mycelium invaded the plate). The cultivation temperature in the case of *Botrytis cinerea* was lower, respectively 21.4ºC which is considered as optimal for the specie (Judet-Correia et al., 2010). For the positive strains the laccase production was monitored further on PDA plates supplemented with different guaiacol concentrations (0.01%, 0.025%, 0.05%, 0.075%, 0.1%, 0.25%, 0.5%, 0.75%, 1%) when the halo formation was daily measured (cm in diameter). The tests were performed in duplicate and the mean values were compared; no significant deviations from the mean were registered in the halo size.

RESULTS AND DISCUSSIONS

In our initial on-plate screening for laccase production (PDA added with 0.04% guaiacol) were tested seven filamentous fungi and six macromycetes, of which only two filamentous fungi and three macromycetes tested positive (Table 2).

Among the six tested macromycetes, only the commercial strains of *Pleurotus ostreatus* and *Agaricus bisporus* have produced distinct brown-reddish halo in the first step screening on medium supplement with 0.04% guaiacol. Comparing the two species, *P. ostreatus* produced a bigger halo under the same cultivation condition and was taken into account for further testing targeting the optimisation of the screening procedure under different guaiacol concentrations (0.01%, 0.025%, 0.05%, 0.075%, 0.1%, 0.25%, 0.5%, 0.75%, 1%).

Table 2. Qualitative results of the on plate initial screening for fungal laccase production on medium supplemented with 0.04% guaiacol

Specie/Strain/Variety	Screening result
Aspergillus clavatus	-
Aspergillus aculeatus	-
Botrytis cinerea	-
Neurospora crassa	-
*Trichoderma sp.*MI2	+
Trichoderma sp. CP	+
Penicillum digitatum	-
Laetiporus sulphureus DD	-
Laetiporus sulphureus B	-
Ganoderma lucidum	-
Agaricus bisporus white variety	+
Agaricus bisporus brown variety	+
Pleurotus ostreatus	++

Legend: (-): negative; (+): 0-3 cm halo; (++): 3-9 cm halo

They were noticed two different pattern groups in the *Pleurotus ostreatus* mycelia development (Figure 1). When using small concentration of guaiacol (0.01% to 0.05%) the plate was invaded by the mycelia after 11 cultivation days at 28°C, while for higher guaiacol concentrations (0.1 to 1%) the mycelial growth was inhibited, and, even after 14 incubation days, the mycelia didn't invade the entire plate. This can be related to the fact that under higher guaiacol concentration more laccase is produced and few species/strains have been reported to have high laccase tolerance (Devasia & Nair, 2016).

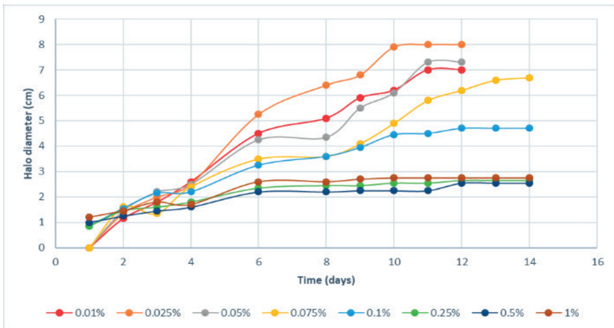

Figure 1. Laccase halo formation evolution for *Pleurotus ostreatus* cultivation at 28°C in PDA supplemented with different guaiacol concentrations

The halo formation followed the mycelial growth evolution and, similarly, two patterns were noticed. In the case of low guaiacol concentrations (0.01% to 0.05%) the halo was not visible in the first incubation day, becoming clearly visible in the third incubation day; from the 10th incubation day, when the maximum was achieved, no significant changes were registered. In the second pattern, of the high guaiacol concentrations (0.1 to 1%), the brown-reddish halo was formed even from the first incubation day (in the first 4 hours), but its development didn't register a major increment starting with the 5th incubation day, being constant till the end of monitoring (14th incubation day). In the case of the 0.075% guaiacol content it was noticed an intermediate pattern between the two groups.

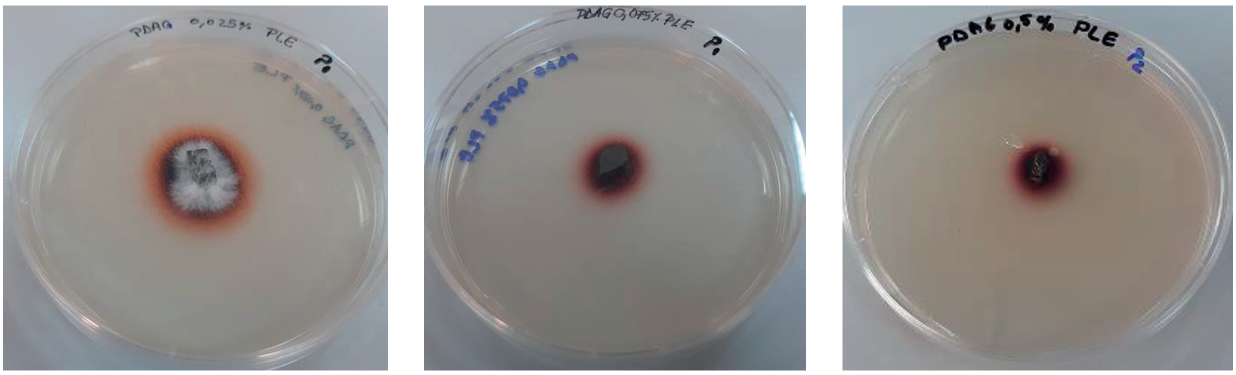

Figure 2. Aspects of the laccase halo formation for *Pleurotus ostreatus* at different guaiacol concentrations in the 3rd incubation day (from left to right: 0.025%; 0.075%; 0.5%)

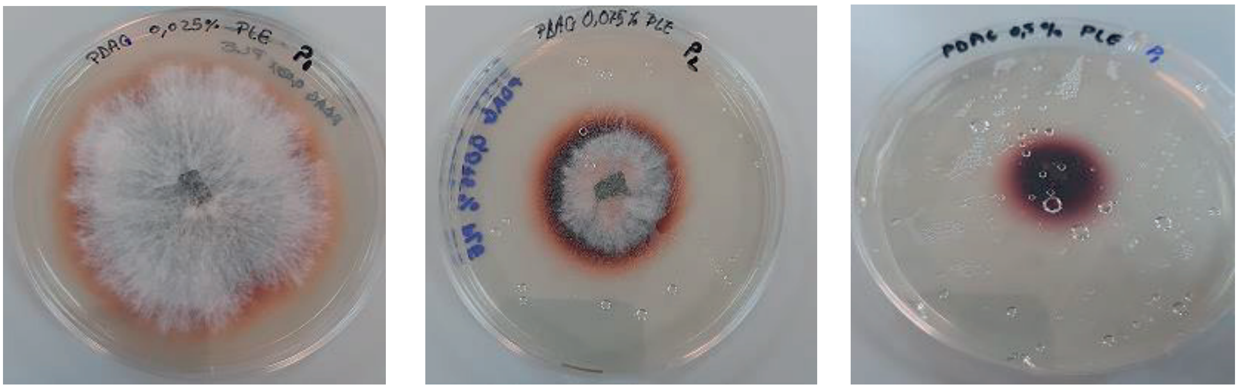

Figure 3. Aspects of the laccase halo formation for *Pleurotus ostreatus* at different guaiacol concentrations in the 8th incubation day (from left to right: 0.025%; 0.075%; 0.5%)

Most of the reported data emphasize that in the case of the plate assay method for fungal laccase test, using guaiacol as substrate in the medium, the brown-reddish zone developed by the isolated strains may appear even from the first incubation day, but can be clearly visible in the 3rd incubation day (Kiiskinen et al., 2004; Kalra et al., 2013; Fu et al., 2013), which is in line with our findings (Figure 2). In the case of *Pleurotus ostreatus* the halo was clearly visible for all tested concentrations, in accordance to other reports; the difference in the halo size between the 3rd and the 8th incubation days can be visualized comparing Figure 2 with Figure 3.

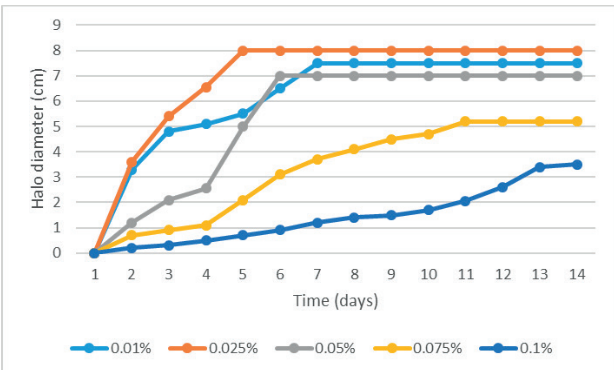

Figure 4. Laccase halo formation evolution for *Trichoderma* spp. MI2 cultivatated at 28°C in PDA supplemented with different guaiacol concentrations

Among the seven tested micromycetes (filamentous fungi), only two isolates of the same genus, namely *Trichoderma*, formed visible halo for the laccase production. This is only partially in line with other authors reports which listed species like *Neurospora crassa, Botrytis cinerea* (Gochev & Krastanov, 2007) or *Penicillium digitatum* (El-Shora et al., 2008) as high potential laccase producers; that may be expalined due to the used strains or to the cultivation conditions.

Both *Trichoderma* isolates were tested further to optimise the screening procedure in different guaiacol concentration. Surprinsingly, when using guaiacol above 0.1% and till 1% no halo formation was detected, while the mycelial growth was not inhibited as was the case of the macromycetal *Pleurotus ostreatus*. It can be noticed that both *Trichoderma* isolates registered similar halo formation evolution for all guaiacol concentrations (Figure 4 and Figure 5).

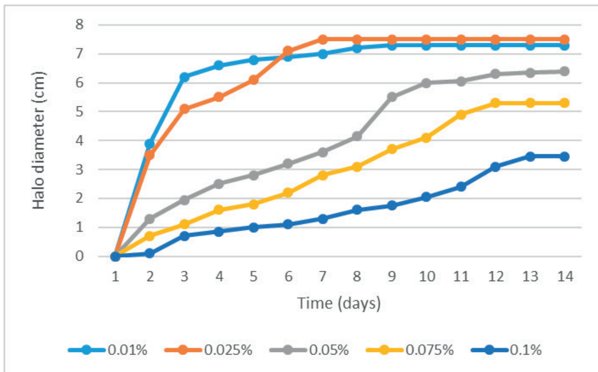

Figure 5. Laccase halo formation evolution for *Trichoderma* spp. CP cultivated at 28°C in PDA supplemented with different guaiacol concentrations

A slight exception was noticed in the case of the 0.05% concentration when the strain MI2 exhibited a pattern closer to the smaller concentration group, while in the case of the strain CP the pattern was closer to the higher concentration group. The higher halos (7-8 cm) were registered for the concentrations of 0.01% and 0.025 %, when the maximum was reached after 6-7 cultivation days. This is in line with data reported by Ahmed & Siddiqui (2015). Aspects of the halo formation at 0.025% for both *Trichoderma* isolates after three cultivation days are visible in Figure 6.

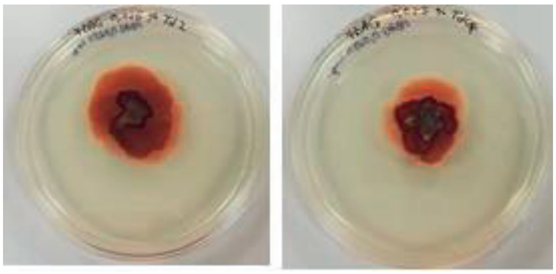

Figure 6. Aspects of the laccase halo formation in the presence of 0.025% guaiacol after 3 incubation days at 28ºC (left: *Trichoderma* spp. MI2; right: *Trichoderma* spp. CP)

Reported data (Ranimol et al., 2018) on *Trichoderma harzianum* suggested as optimal guaiacol concentration for laccase on-plate screening as 0.05%, which is only partially in accordance with our results.

CONCLUSIONS

In our attempt to screen among different fungal species for laccase production, we have identified positive strains belonging to macro or micromycetes groups, respectively two strains of *Trichoderma* spp. isolated from soils, one variety of *Pleurotus ostreatus* and two of *Agaricus bisporus* originating from supermarket wastes. Among the macromycetes, *Pleurotus ostreatus* exhibited the highest potential for laccase production, while in the *Trichoderma* spp. strains, their potentials were close.

In terms of guaiacol concentration used in the screening, some conclusions are to be taken into account, depending on the fungal group. In the case of the macromycete group, respectively *Pleurotus ostreatus*, using guaiacol concentration higher than 0.1% is inhibiting the fungal growth, as well as the halo–formation; this may be correlated to the strain tolerance to the presence of laccase in the medium. Also, in small guaiacol concentrations (0.01-0.05%) the halo formation is clearly visible in the third cultivation day, while for higher concentrations (0.1-1%) in the first 4 hours the halo is visible. In the case of *Trichoderma* spp. isolates, when using guaiacol above 0.1% and till 1% no halo formation was detected, while the mycelial growth was not inhibited; the higher halos sizes were registered for the concentrations of 0.01% and 0.025%, when the maximum was reached after 6-7 cultivation days.

For both fungal groups, when on-plate screening is performed it is recommended to be used lower guaiacol concentrations, starting with 0.01% to 0.075%. Higher guaiacol concentration (0.1% to 1%) may induce the laccase formation in a very first step, but may inhibit the mycelial growth and are recommended only when screening for fungal strains tolerant to high laccase formation. Guaiacol was confirmed, in small concentrations (0.01-0.05%), as useful indicator when screening for both laccase producers and laccase tolerant fungi of bioremediation use.

Further investigations are taken into account on how guaiacol concentration induces the laccase production and tolerance under fungal submerged culture.

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STUDY ON STRONGYLE INFECTION AND OTHER ASSOCIATED PARASITES IN SHEEP, IN SOUTHERN AREA OF ROMANIA

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Abstract

Parasitism of diferent strongylid nematodes belonging to the families Trichostrongylidae, Strongylidae and Ancylostomidae on sheep, are widespread in all regions of animal breeding, but especially in areas with a humid climate. As they result in drastic decreases in production of adult animals and growing delays in young animals, imposing costly prophylaxis measures, these parasitic diseases have a significant economic impact. While studies in helminthology are directed towards more in-depth perspectives, such as DNA modifications of parasites, the continuous monitoring of occurrence and prevalence of these parasitic species remains important. In addition, because there are few reports on gastrointestinal parasites of ruminants and especially of sheep in our country, the present study aims at investigating the occurrence of strongyle infections in sheep, in Southern Romania. For this, a coproparasitological study was carried out on a total number of 424 sheep, out of a population of 3256 animals, originating from six farms from this region. A flotation technique was used to detect helminth eggs; however, protozoa oocysts, when detected, were also registered; additionally a Baermann method, for detection of lungworm larvae was performed. The findings indicate the following infections, as follows: strongyles 65.1%; Moniezia spp. 18.9%; Eimeria spp. 14.4%; Protostrongylidae 7.1%, Dictyocaulus filaria 2.1%. The findings highlight the high occurrence of strongyle infections, but also of other parasitic species with significant impact on the both animal health and their productivity and highlight the importance of a proper parasitological control to be applied in sheep farms.

***Key words**: sheep strongyles, prevalence, Southern Romania.*

INTRODUCTION

Parasitism of digestive strongyle on sheep is widespread and causes important economic losses, with a significant impact on the sheep industry worldwide, including Romania. Recent studies in the East (Kumar et al., 2015; Mitrea, 2011; Rajpoot et al., 2017), Middle-East (Gholami et al., 2015; Sharifdini et al., 2017) and France (Arece-García et al., 2007; Mokhtar et al., 2009) have shown a greater interest towards this subject, thus enriching the knowledge in the field. While, surely, parasitic infections worldwide will be based, more or less, on the same issues, it is the prevalence of some and not other, and the particularity of certain variables that determines the degree and specifics of parasitism in a country or area. Furthermore, while current studies of helminthology have become increasingly focused on various DNA manipulations of parasites (Horak, 2019; Marchiondo et al., 2019; Wang et al., 2013), there are still of high interest epidemiological studies, which are the basis for developing sustainable parasitological control programs.

It is widely acknowledged that gastro-intestinal strongyle infections cause important economic costs to sheep breeders in terms of production, reproduction, weight gain and, not least, mortality (Odoi et al., 2007).

Evidently, the impact on animal welfare is exponential. In this context, early diagnosis and farm management, understood as using proper prophylactic measures, are vital to maintain the lowest levels of helminths' incidence.

While, a significant number of studies have recently reported on the occurence and prevalence of endo-parasites in Romanian horses (Madeira de Carvalho et al., 2008; Covasa and Miron, 2011; Ionita et al., 2013; Cernea et al., 2015; Buzatu et al., 2014; 2016; Morariu et al., 2016), information about the epidemiology of gastro-intestinal parasites in sheep is still parsimonious.

Due to the fact that our country ranks the 4th place in the EU regarding sheep flocks, it is necessary for continuous updating studies in the field. The present study aimed to determine the presence of gastro-intestinal strongyles and other associated endo-parasites on sheep in South of Romania, as well as the factors influencing the presence of these, in order to characterize the pattern of parasitism in the area.

MATERIALS AND METHODS

The study was carried out on a representative batch of 424 sheep, from a population of 3256 sheep, between the years 2012-2016. The investigated farms consisted of three agrozootechnical units (AIC, FS, OM) and three private households (SC, AM, BA), in Southern Romania.

The batch comprised approximately 85.14% females (lactating, pregnant), aging over 2.5 years old and the rest, lambs, male and female youth, and adult males; the age of animals varied between 2.5 months and 5 years . The sheep included in the study were of the following breeds: Tigae, German Blackhead, Merinos Palas, Teleorman Carabas, and Texel.

Fecal samples were collected from individual animals, in 3 series, in all seasons.

The following methods and analyses were performed:

- coproparasitological investigations for detecting eggs of digestive parasites werecarried out, by flotation (Willis technique). Aditional, a larvoscopic technique (Baermann method) was performed (Ionita and Mitrea, 2013). These investigations were performed in the Laboratory of Parasitology of the Faculty of Veterinary Medicine, USAMV Bucharest. The information was coded into SPSS (Statistical Package for Social Sciences), with a confidence interval of 95%. While each of the homesteads had differentf lock numbers, equivalent examples were taken (cca 70 for each ranch) to maintain a strategic distance from critical varieties in results.
- anatomopathological analyses, with samples taken promptly after evisceration and necropsies from the small and thick intestine, and rennet.

The parasites' prevalence was evaluated using the Frequencies and Crosstabs commands in SPSS. The factors potentially influencial of gastrointestinal infections were explored through Correlations commands and One-Way ANOVA analyses.

Discussions were held with the owners and farm administrators about the frequency of prophylactic treatments anytime within the period of research.

The variables introduced into the statistical analysis to evaluate the parasites' incidence consisted of: the sheep age range (1-18 months; 19-60 months) and race; the season; and year in which the samples were collected; the use of prophylactic treatments; the presence of associated parasitism; the farm type (agrozootechnical, individual household).

RESULTS AND DISCUSSIONS

Overall, a proportion of 69.8% (296/424) of the animals included in the study were positive for at least one parasite infection (Table1).

Table 1. Parasite infection levels per each farm included in the study

Animals	Prevalence of parasite infection in sheep by farm					
	AIC	FS	OM	SC	AM	BA
Nr. Sampled	70	70	70	74	70	70
Nr. (%) positive	49 (70%)	18 (25.71%)	35 (50%)	64 (86.48%)	60 (85.71%)	70 (100%)

The coproparasitological examinations revealed the presence of protozoan oocysts, eggs of gastrointestinal helminth parasites and larvae of lungworms (Figures 1-5), as following: GI-strongyles, *Eimeria* spp., *Moniezia* spp., *Protostrongylus* spp., *Dictyocaulus filaria*.

Figure 1. Digestive strongyle (eggs) in sheep (ob. 10x)

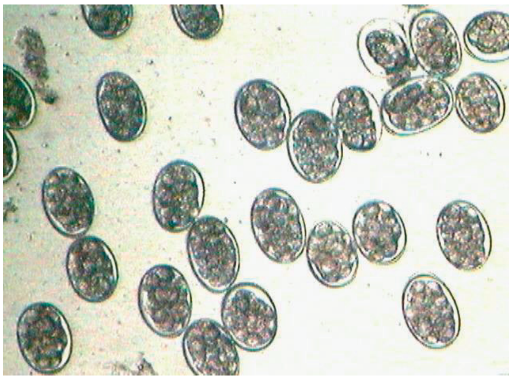

Figure 2. Heavy infection with digestive strongyle (eggs) in sheep (ob. 20x)

Figure 3. Mixt infection: digestive strongyle (eggs) and *Eimeria* (oocysts) in sheep (ob. 20x)

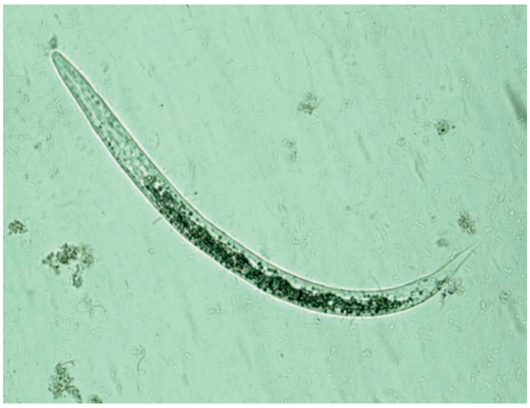

Figure 4. Larva of *Dictyocaulus filaria* (lungworm) in sheep (ob. 10x)

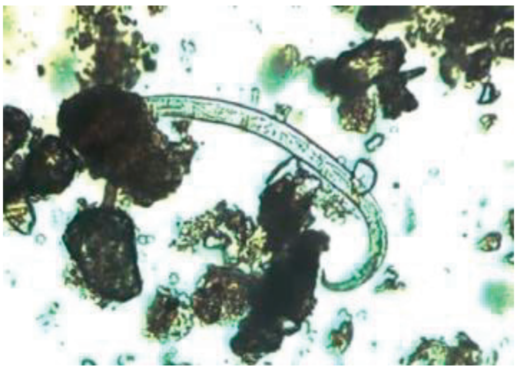

Figure 5. Protostrongylid larva (lungworm) in sheep (ob. 10x)

The prevalence of the parasite species identified in sheep is presented in Table 2.

Table 2. Prevalence of parasite species in the sheep included in study (frequencies and percentages)

Parameter/ locality	**Parasite species identified**				
	GI-strongyles	*Moniezia* spp.	*Eimeria* spp.	*Protostrongylids*	*Dictyocaulus filaria*
Total Number of infected animals	276	80	61	30	9
Percentages	65.1%	18. 9%	14.4%	7.1%	2.1%
	Number of positives animals per farm (frequencies and percentages)				
AIC (n=70)	47 (67.1%)	2 (2.85%)	17 (24.3%)	0 (0%)	4 (5.79%)
FS (n=70)	16 (22.9%)	13 (18.6%)	5 (7.1%)	2 (2.9%)	0 (0%)
OM (n=70)	32 (45.7%)	3 (4.3%)	10 (14.3%)	0 (0%)	3 (4.3%)
SC (n=74)	64 (86.5%)	42 (56.8%)	0 (0%)	7 (9.5%)	0 (0%)
AM (n=70)	52 (74.3%)	13 (18.6%)	18 (25.7%)	8 (11.4%)	0 (0%)
BA (n=70)	65 (92.8%)	7 (10.0%)	11 (15.7%)	13 (18.6%)	2 (2.85%)

Concerning the incidence by age, adults presented infections in proportion of 65.02% compared to the youth, in 98.36% of the specific herds. The proportions are statistically significant (p = -255) and illustrate that parasitism is inversely proportional to age: the younger the specimens, the greater the likelihood of parasites existence.

This result is consistent with the existing literature, including recent studies in Romania that emphasize the predisposition of youth to parasitism, but to varying degrees: in a study conducted in Cluj County, Negrea et al. (2013) reported the presence of strongyles in the range of 72.7% in youth and 65% in adults. In our study, the youth presented infections with strongyles in proportion of 95.1%, and adults, 60.1%, data which are correlated significantly (p = -258).

The periods of spring (26%, N = 424; df = 3) and autumn (20%, N = 424; df = 3) had the most frequent cases of parasitism, especially infections with strongyles: 22.6% spring, 19.3% autumn. In summer and winter, parasitic infections were generally identified in proportion of 14%, respectively 10%, and with strongyles particularly, in proportion of 13.2%, respectively 9.9%. The correlations in this respect were statistically significant (p = -253).

Of the cases of parasitism prevalent, the male youth was affected 100%, the female youth, in

96.77%, and the adult females, in 64.85% (df = 1). The results were significantly influenced by the type of farm: the individual households had very high levels of infection (65.5%), one of them reaching 100% degree of infection, while the agrozootechnical farms showed lower levels (34.5%, SD = 0. 47).

Prophylactic treatments were administered to 24.3% of the specimens (n = 424; SD = 0.42), however there were large variations between farms, as in the case of parasites' incidence: agrozootechnical farms performed prophylactic treatments in proportion to 17.45%, while the individual households, 6.83% (SD = 0.45). The FS farm differentiated itself as a role-model, with the lowest level of parasitism, as well as with the highest frequency of prophylactic treatments. In addition, the use prophylactic treatments represented a significant variable influencing parasitim (p =.157).

The incidence of parasites in the sample was, as follows: strongyles - 65.1%, *Moniezia* spp. - 18.9%, *Eimeria* - 14.4%, *Protostrongylus* spp. - 7.1%, *Dictyocaulus filaria* - 2.1% (N = 424).

Among the detected parasitoses, numerous infections were mixed: while there was a significant percentage of simple infections with strongyles (31%) and *Moniezia* (15%), there were important proportions of mixed infections of strongyles with *Moniezia* spp. (15%), strongyles and *Eimeria* (12%), strongyles and *Protostrongylus* (4%); the rest being infections with *Eimeria* and *Protostrongylus* spp. (2%); and strongyles with *Dictyocaulus filaria* (2%, N = 424).

In a study conducted on sheep flocks from Northern and Southern Romania, Mitrea et al. (2008) indicated the presence of strongyles in relation to other parasitic populations, in a range of 71.5% to 92.8%; and *Moniezia*, between 14.2% and 21.4%. The results of the present study are close to these values, through the presence of strongyles in proportion of 65.1%; and converge in what concerns *Moniezia* spp., with an incidence of 18.9%.

At the same time, the results of our research converge with those highlighted by Mitrea et al. (2008) additionally in what regards the prevalence of parasitic associations with two species: the cited study indicates an interval between 14.28% to 35.7%, and our empirical approach describes a percentage of 35% of the sample. However, our research did not reveal associations with more than two parasitic species, nor any species other than those discussed, and the prevalence of *Eimeria* is different (up to 50%) (Mitrea et al., 2008), compared to 14.4%, in the present study.

The gender was a significant variable at the level of the whole sample. As in the recent literature in our country, this variable has not been signalled as statistically significant in sheep, but rather in other geographical areas - such as, for example, sheep genderin the Punjab area (Singh et al., 2017), future studies will have to further assess the validity of these significant values on larger samples from Romania.

Similarly, performing prophylactic treatments was another significant variable in the influence on parasitism, which has not been reported as significant in sheep in Romania, but was indicated as such in countries such as Brazil (Machado et al., 2019).

While age and prophylactic treatments are significantly correlated with parasitism level, the type of farms remains variable with the strongest impact (Mitrea, 2002), a fact that emerges from the highest level of correlation. Thus, as Indre et al. (2011) outlines, parasitic situations differ greatly from one farm to another. FS stood out as a standard model in terms of the low level of parasitism (25.7% of its own sample) (Table 1), as well as in the use of prophylactic treatments (51.42%). All variables indicated here as significant are predictors of parasitism in sheep in analyzed sample in the range 34% (R^2 = .377).

CONCLUSIONS

To conclude, in the interval between 2012-2016, what seems to have been specific for the Southern area of Romania is the prominent presence of strongyles, in both single and mixed infections and the most important variable influencing this was the type of farm sheltering the animals.

Beyond the variables discussed in the analysis, there is, undoubtedly, a management component of the grazing and sheltering of the sheep that may determine the presence or absence of parasitism; and which can justify the different levels of parasitism in the 6 farms. The fact that there is a significant and strong correlation between the types of farms sheltering the sheep and parasitism can be attributed to additional prophylactic measures, unaccounted for by our approach.

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ALLELOPATHIC EFFECTS OF *Grevillea banksii* R. BR. LEAF EXTRACTS AND ITS RHIZOSPHERIC SOIL ON GERMINATION AND INITIAL GROWTH OF THREE AGRICULTURAL CROPS IN MADAGASCAR

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Abstract

G. banksii R. Br. is a widespread species forming dense populations in the Eastern part of Madagascar. The aim of this study was to investigate the allelopathic effects of G. banksii on seeds germination and initial growth of three agricultural crops: rice (Oriza sativa L.), maize (Zea mays L.) and bean (Phaseolus vulgaris L.). The effects of leaf (leaf powder and aqueous leaf extracts obtained after shaking during 24 and 48 h) and rhizospheric soil (soil powder and aqueous soil extract shaken during 72 h) of G. banksii on seeds germination, seedling length of each tested plant were described in vitro. Results showed that the rhizospheric soil of G. banksii activates the germination of bean and maize seeds as well as the seedling length development of all crop species but inhibit rice seeds germination. However, all aqueous leaf extract inhibit seeds germination and the shoot and root length development of the three crops species tested, especially for rice and maize roots length development. This investigation therefore comparatively reveals that the leaves extract of G. banksii has more allelopathy than rhizospheric soil in seed germination and initial growth of agricultural crops tested. The results suggest some cautions when using the shoots of G. banksii as plant cover in farming systems.

Key words*: allelopathic, crop plants, invasive plant, seed germination, root and shoot elongation.*

INTRODUCTION

G. banksii R. Br. (Proteaceae) is a plant species originated in Australia and introduced in the Eastern part of Madagascar in the 1950s in order to restore forest cover and to limit land degradation/erosion in this region (Tassin, 1995). After a few years of establishment, this exotic species was identified as an invasive species in Eastern part of Madagascar (Binggeli, 2003).This species is known for its
Many research results have suggested that exotic invasive plants release some allelopathic substances into the environment (Osvald, 1948; Fletcher and Renney, 1963; Abdul Wahab and Rice, 1967; Kanchan and Jayachandra, 1979;

capacity to spread rapidly especially on disturbed areas previously occupied by forest ecosystems. Moreover, *G. banksii* may affect the regeneration of native plant species by inhibiting soil biota which may have significant roles in plant development (Andrianandrasana et al., 2014). In spite of this, the farmers in the region use its biomass in farming through slash and burn farming practices. But, the interaction involved between crop plant species and this invasive plant is not well understood yet (El Ghareeb, 1991; Vaughn and Berhow, 1999; Ridenour and Callaway, 2001; Stan et al., 2018) and exploit this phenomenon to attribute their dominance success and their competitiveness (Ridenour and Callaway, 2001; Stinson et al., 2006; Jarchow and Cook, 2009). Thus, allelopathy is defined as any direct or indirect, positive or negative effect of a plant, including microorganisms, on neighbouring species through the release of biochemical compounds (Rice, 1984) that could be present in whole plant or only in some organs, like roots, rhizomes, stems, leaves, fruits and/or seeds (Zeng et al., 2008). Root exudates represent one of the largest direct inputs of plant chemical elements into the rhizosphere (Bertin et al., 2003).

Many allelopathic compounds affect crops development. Most of them are described as the main actors in the inhibition of seeds germination, overall growth and plant nutrients' uptake (Rizvi et al., 1999; Marwatand Khan, 2006). For example, Ejaz et al. (2004) found that allelopathic compounds produced by *Eucalyptus* decrease the cotton germination rate. Balicevic et al. (2015) have reported that the invasive plant, *Solidago gigantea*, decreased the germination of seeds and development of seedlings of carrot, coriander and barley.

The aim of this study was to assess allelopathic effects of leaf and rhizospheric soil of *G. banksii* on seeds germination rate and initial growth of three crops species *in vitro*.

MATERIALS AND METHODS

Plants materials and soil collection

Leaves and rhizospheric soil were collected from mature individuals of *G. banksii* growing in the Eastern part of Madagascar (18°57'48.0''S, 048°45'51.3''E). The collected samples were brought into the Laboratory of Environmental Microbiology of National Center of Environment Research (CNRE) Madagascar.

Seeds of rice (*Oriza sativa* L.), maize (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.) were used as test plants in all experiments to assess the allelopathic effects of *G. banksii*. Seeds of *O. sativa* (variety Botrafotsy) and *Z. mays* were taken from farmers' stock in the eastern part of Madagascar.

Seeds of *P. vulgaris*, variety Ranjonomby, were provided by the FOFIFA (National Center for Applied Research on Rural Development in Madagascar).

Aqueous extract preparation

Leaf of *G. banksii* was dried at room temperature and cut into 1-2 cm pieces. Leaf was pounded using electrical stainless material. Aqueous leaf extracts were prepared by soaking 10 g leaf powder of *G. banksii* with 200 ml sterile distilled water. Each container was shaken separately for 24 and 48 hours at room temperature. The resulting aqueous extracts were filtered with Whatman No.1 filter paper. Rhizospheric soil extract was prepared by soaking 10 g of soil in 200 ml sterile distilled water at room temperature for 72 h and filtered.

Seed germination and seedling growth

Effect of aqueous extracts. Five millilitre (5 ml) of each aqueous extracts types (two leaf extracts and one soil extract) were tested on the three test plant seeds (rice, maize and bean) which were respectively deposited on filter paper contained insterilized Petri dishes. Sterile distilled water was used as control. For each treatment, four replicates, each with 10 seeds, were made. The Petri dishes were incubated at 25°C. After 5 to 10 days, seeds germination and initial growth (shoot and root length) of each plant were noted.

Effect of leaf and soil powder. Five grams of leaf powder and 2 g of rhizosphere soil powder were placed separately in Petri dish and topped with a single sheet of filter paper. Ten seeds of crop species were placed on it. The dishes were moistened with 10 ml sterile distilled water. For the control, fine pieces of filter paper were used. The bioassay was run as mentioned above.

Data analyses

The results were quantified as germination capacity, root and shoot length development. Germination percentage G (%) was calculated using the following formula:

$$\mathbf{G\ (\%) = \frac{N}{Nt}\ X\ 100}$$

Where: **N** is germinated seeds in each treatment and **Nt** - number of seeds used in bioassay.

The relative inhibition (**I**) or stimulation (**S**) of seed germination and shoot and root length development affected by the allelopathic substance was calculated according to Chung et al. (2001) and Ladhari et al. (2013) as following:

$$\mathbf{I\ (\%)\ or\ S\ (\%) = \frac{E - C}{C} X\ 100}$$

Where: **E** is extract (growth parameter measured in presence of *G. banksii* leaf or soil extract and powder) and **C** - control (growth parameter measured in presence of sterile distilled water).

All statistical analyses were done using XLSTAT 2008 software. Differences among the treatment means were assessed according to ANOVA test.

Significant differences among the means were found through Fisher (LSD) significant difference test at $P < 0.05$.

RESULTS AND DISCUSSIONS

1. Allelopathic effects of leaf of *G. banksii* on crop plants species

1.1. Germination percentage

The effects of *G. banksii* leaf extracts on the germination capacity of rice, maize and bean are shown in Table 1. The results showed that leaf affected seed germination of rice, maize and bean except for the 24 h leaf extract on rice and leaf powder on maize. Notable inhibition was found on rice germination with 48 h leaf extract (-24.88%) and leaf powder (-36.15%). However, rice seed germination stimulation was found with 24 h leaf extract (+23.94%) and a little stimulation on maize germination by both 48 h leaf extract and leaf powder was recorded (Figure 1).

Table 1. Seeds germination capacity (%) of rice, maize and bean in presence of leaf of *G. banksii*

Treatments	Seeds germination capacity (%)		
	Rice	Maize	Bean
24 h leaf extract	82.50 ± 17.50 (a)	72.50 ± 17.50 (b)	85.00 ± 7.50 (b)
48 h leaf extract	50.00 ± 5.00 (a)	95.00 ± 5.00 (a)	90.00 ± 5.00 (ab)
Leaf powder	42.50 ± 4.20 (a)	97.50 ± 3.75 (a)	95.00 ± 5.00 (ab)
Control	66.56 ± 5.31(a)	93.75 ± 9.39 (a)	98.00 ± 2.00 (a)

Means with the same letter in a column are not significantly different at $p < 0.05$ according to Fisher's LSD test.

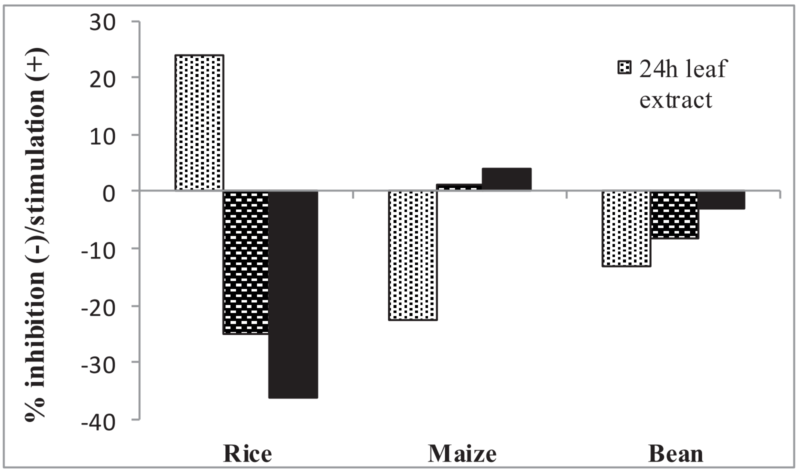


Figure 1. Inhibition (-)/stimulation (+) of germination seeds of rice, maize and bean in presence of leaf of *G. banksii*

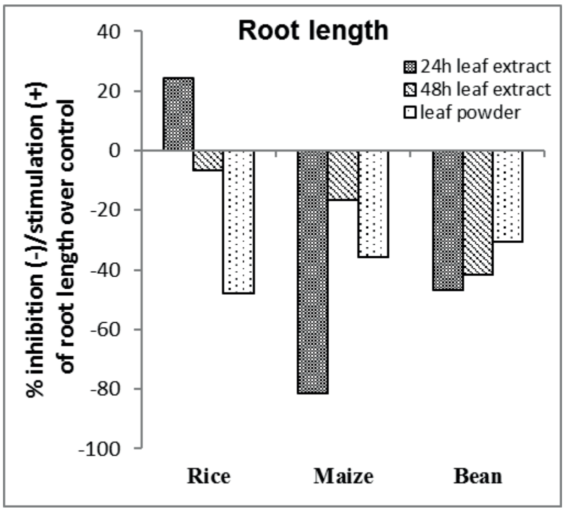
1.2. Root and shoot length development

Effects of leaf of *G. banksii* on root and shoot length development of rice, bean and maize are shown in Table 2. Root and shoot length development of rice, maize and bean were reduced by leaf of *G. banksii* except for the 24 h leaf extract on rice root length development. The highest inhibition was found on maize root length development with 24 h leaf extract (-81.44%) and on maize shoot length development with 24 h and 48 h leaf extracts (-100%) (Figure 2). Generally, the leaf of *G. banksii* proved to be toxic to roots and shoots length development of the three tested plant species.

Table 2. Root and shoot length development of rice, maize and bean in presence of leaf of *G. banksii*

	Treatment			
	24 h leaf extract	48 h leaf extract	Leaf powder	Control
Root length (cm)				
Rice	2.21 ± 1.46 (a)	1.66 ± 1.66 (a)	0.93 ± 0.90 (a)	1.78 ± 1.65 (a)
Maize	1.06 ± 0.46 (c)	4.79 ± 0.48 (ab)	3.68 ± 0.56 (b)	5.73 ± 1.65 (a)
Bean	3.48 ± 1.47 (b)	3.83 ± 0.97 (b)	4.55 ± 1.35 (ab)	6.55 ± 1.24 (a)
Shoot length (cm)				
Rice	0.78 ± 0.50 (a)	0.63 ± 0.62 (ab)	0.01 ± 0.02 (b)	0.78 ± 0.12 (a)
Maize	0.00 ± 0.00 (b)	0.00 ± 0.00 (b)	0.13 ± 0.02 (b)	1.14 ± 0.72 (a)
Bean	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)

Means with the same letter in a line are not significantly different at $p < 0.05$ according to Fisher's LSD test.



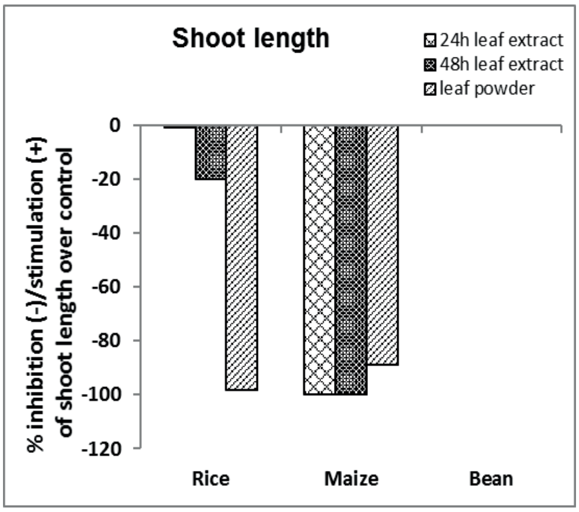


Figure 2. Inhibition (-)/stimulation (+) of root and shoot length development of rice, maize and bean in presence of leaf of *G. banksii*

2. Allelopathic effect of the rhizospheric soil of *G. banksii* on crop plants species

2.1. Germination percentage

Compared to control treatment, soil powder and soil extract did not significantly ($p < 0.05$) reduced seeds germination percentage of rice, maize and bean (Table 3).

Besides, inhibition/stimulation analyses revealed that maize seeds germination was stimulated by rhizospheric soil powder and rhizospheric soil extract respectively +6.66% and + 4%.

But, rice seed germination was inhibited by rhizospheric soil powder and rhizospheric soil extract respectively -17.37% and -2.34%. However, bean seeds germination was stimulated with rhizospheric soil powder (+2.04%) but inhibited by rhizospheric soil extract (-5.80%) (Figure 3).

Table 3. Germination capacity (%) of seeds of rice, maize and bean in presence of rhizospheric soil of *G. banksii*

Treatment	Seeds germination capacity (%)		
	Rice	Maize	Bean
Soil powder	55.00 ± 4.50 (a)	100.00 ± 0.00 (a)	100.00 ± 0.00 (a)
Soil extract	65.00 ± 10.00 (a)	97.50 ± 3.70 (a)	92.31 ± 7.70 (a)
Control	66.56 ± 5.30 (a)	93.75 ± 9.30 (a)	98.00 ± 2.00 (a)

Means with the same letter in a column are not significantly different at $p < 0.05$ according to Fisher's LSD test.

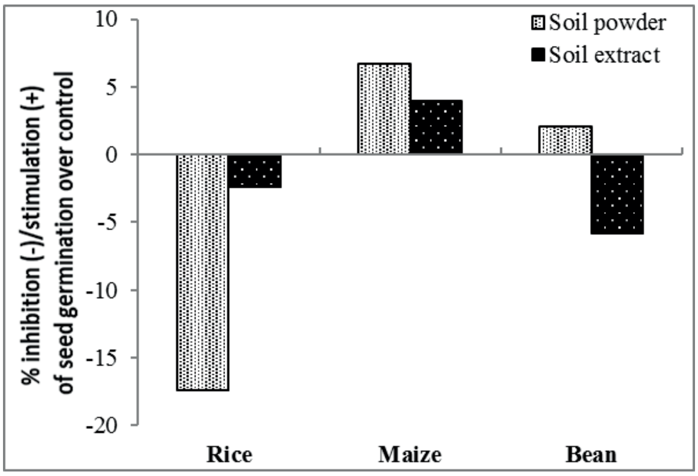


Figure 3. Inhibition (-)/stimulation (+) of germination seeds of rice, maize and bean in presence of rhizospheric soil of *G. banksii*

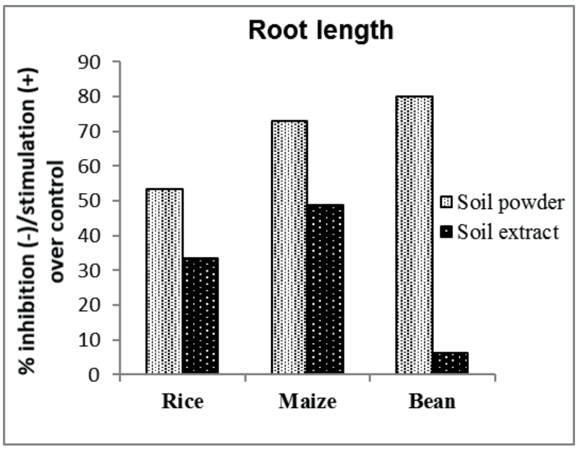
2.2. Root and shoot length development

Root length development of rice, maize and bean was significantly ($p < 0.05$) stimulated by both rhizospheric soil powder and extract. Shoot length development of maize was also stimulated by these two treatments (Table 4). The highest stimulation was found on bean root length development with rhizospheric soil powder (+80.03%) and on maize shoot length development with the same treatment (+228.94%) (Figure 4).

Table 4. Root and shoot length development of rice, maize and bean in presence of rhizospheric soil of *G. banksia*

	Treatment		
	Rhizospheric Soil extract	Rhizospheric Soil powder	Control
Root length (cm)			
Rice	2.73 ± 0.62 (a)	2.38 ± 0.70 (a)	1.78 ± 0.18 (a)
Maize	9.91 ± 0.98 (a)	8.53 ± 0.81 (a)	5.73 ± 1.65 (b)
Bean	11.79 ± 1.86 (a)	6.96 ± 1.58 (b)	6.55 ± 1.24 (b)
Shoot length (cm)			
Rice	0.59 ± 0.56 (a)	0.73 ± 0.37 (a)	0.78± 0.12 (a)
Maize	3.75 ± 0.85 (a)	1.93 ± 0.75 (b)	1.14 ± 0.72 (b)
Bean	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)

Means with the same letter in a line are not significantly different at $p < 0.05$ according to Fisher's LSD test.



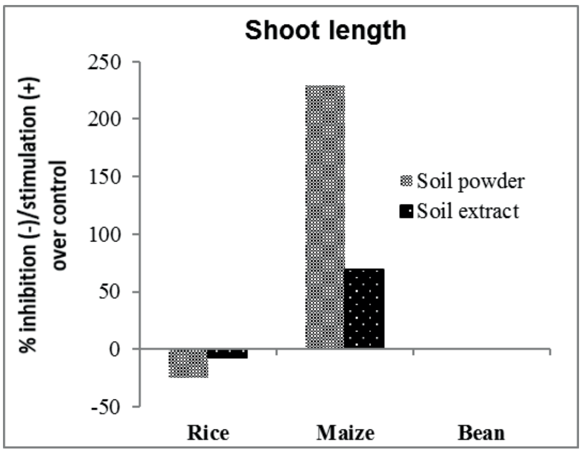


Figure 4. Inhibition (-)/stimulation (+) of root and shoot length development of rice, maize and bean in presence of rhizospheric soil of *G. banksii*

In this study, leaf of *G. banksii* and its rhizospheric soil were used to evaluate their effect on seeds germination and growth of both roots and shoots of rice, maize and bean in vitro. Our results showed that, the allelopathic effect of this exotic plant was varied according to the aqueous extract type (leaf extract and rhizospheric soil extract) and tested plant species. Thus, the response of germination, seedling growth of test plant species towards the same extract (Hisashi et al., 2009) and the toxicity of allelochemical in different part of same plant (Hussain et al., 2010) are variables.

Firstly, all aqueous leaf extracts of *G. banksii* were negatively affected the percentage of seed germination and root and shoot length development of all tested agricultural species. Lara-Núñez et al. (2006; 2009; 2015) explain this phenomenon that allelochemical can alter enzymatic activities during seed germination and radicle growth. Indeed, the high inhibition of seed germination was found on rice with 48h leaf extract.

The results indicate that by increasing the shaken duration, the efficiency of extracts is also increased, especially on rice seed germination. These findings agree with those of Barkat et al. (2010), Hussain et al. (2010) and Ehsan et al. (2012). Contrary for bean, shaken leaf extract during 24 h was more inhibitory than 48 h leaf extract. This might be due to denaturation of phytotoxic substances capable to inhibit particularly bean seed germination. Samreen et al. (2009) and Batlang and Shushu, (2007) suggested that allelopathic stress depends upon concentration of allelopathic material.

Generally, plant litter is known to increase soil fertility during decay. However, our results showed that leaf of *G. banksii* reduce remarkably the seed germination of rice. Similar results were also reported in other studies. For example: *Ageratina adenphora* litter reduced growth of *Lantana camara* (Kaul and Bansal, 2002). Moreover, leaf extracts of *G. banksii* inhibit shoot elongation of maize and rice, respectively -100% and -98.3%. Many studies explained that decrease in root and shoot length development may be attributed to inhibiting or reducing rate of cell division and elongation due to the presence of allelochemicals compound which inhibit probably the hormones such as gibberellin and indoleacetic acid function in the plant species (Tomaszewski and Thimann, 1966).

Secondary, allelopathic effect of rhizospheric soil of *G. banksii* was evaluated using an aqueous extract and powder of soil on germination and shoot length development of three crops plants. Samedani et al. (2013) explain that allelochemicals activities in soil are depend on complex interactions betwen soil and plant factors. In this way, soil properties are the dominant factors determining the activity of allelochemicals in soil (Inderjit, 2002). As other species of Proteaceae family, *G. banksii* has proteoid roots (Purnell, 1960; Andrianandrasana et al*.,* 2014). This root type is known for its ability to produce acids, such as malates and citrates (Dinkelaker et al*.,* 1995; Shane and Lambers, 2005) and enzymes such as phosphatases (Miller et al., 2001; Gilbert et al., 2000) into the rhizosphere. This root type may affect rhizospheric soil properties of *G. banksii* and subsequently affects germination and growth of other plant species. Our results showed that rhizospheric soil of *G. banksii* inhibited rice and bean seeds germinations but stimulated maize seed germination. Besides, all rhizospheric soil generally activated shoot elongation on the three agricultural crops tested. Therefore, the stimulation growth of crop species by rhizospheric soil could probably be due to the induction of growth promoting hormones that were described by Tomaszewski and Thimann (1966).

CONCLUSIONS

In summary, our results demonstrated that allelopathic effect of *G. banksii* as inhibitory or stimulatory was depending on the extract and on the/or crop species. Indeed, the degree of seeds germination and plant initial growth inhibition were higher in aqueous leaf extract of *G. banksii* than rhizospheric soil on the three crops species tested. Also, rice seed germination was more affected than maize and bean. Remarkable stimulation was observed in maize development with rhizospheric soil. However, this study needs further evaluation at field level. Also, allelopathic potential of *G. banksii* will be tested to it herbicidal activity on weeds species associated with crop plant.

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FORMATION OF THE YIELD AND SEED QUALITIES OF POTATO IN THE NURSERY OF BASIC SEED PRODUCTION UNDER THE CONDITIONS OF THE SOUTH OF UKRAINE

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Abstract

The article presents experimental data on yield, yield structure and quality of seed potatoes of the varieties Skarbnitsa, Levada and Yavir, depending on fertilizer and treatment with growth regulators Emistim S, Regoplant and Stimpo. On average, over three years of research, the enrichment of $N_{45}P_{45}K_{45}$ and $N_{90}P_{90}K_{90}$ increased the yield on 5.45 and 7.09 t ha^{-1} (37 and 48%). With background nutrition $N_{45}P_{45}K_{45}$, growth regulators increased yields from 3.6% to 21.7%, depending on the variety and regulator. Fertilizers and growth regulators increased the dry matter content in tubers on 0.8 and 1.1%, starch - on 0.4 and 0.9%. Due to the use of fertilizers, the content of vitamin C decreased on 3.9 and 7.4%, nitrates increased on 30.1 and 60.4%. The maximum productivity of the early variety Skarbnitsa and medium early Levada was formed with the combined use of mineral fertilizers in a dose of $N_{45}P_{45}K_{45}$ with the Regoplant growth regulator treatment - 21.85 and 22.12 t ha^{-1}. When growing a mid-season variety Yavir, there was a need to use a dose of $N_{90}P_{90}K_{90}$ fertilizers without growth regulator treatment, the yield was 22,65 t ha^{-1}.

Key words: *fertilizers, growth regulators, potato, seed productivity, varietal qualities.*

INTRODUCTION

The best way to obtain seed potatoes in southern Ukraine that are less susceptible to degeneration due to high air temperatures is to plant summer freshly harvested tubers in a two-crop culture (Vozhegova et al., 2014a). Insufficient frost free period and the absence of irrigation in the north and center of the country do not allow the use of the two-crop method for the production of seed potatoes. Therefore, in these regions and in the South of Ukraine, in most households, seed potatoes are grown according to the traditional scheme of seed production - only during spring planting and harvesting under biological maturity of tubers. The development of a complex of measures to improve the productivity of seed potatoes in this scheme is relevant.

For seed potato technology to be considered expedient, it must ensure high yields, seed productivity and quality of the harvest. It is possible to solve this aim while providing all the needs of the plant, for which many factors need to be taken into account, as well as the use of complex growth regulators as an additional source to increase productivity and crop quality.

The use of multicomponent growth regulators can increase field germination, survival, increase plant resistance, allow them to accumulate more tops and roots (Calvo et al., 2014; Jardin, 2015; Usha et al., 2009). Chemical and biological growth regulators are also used in potato production to accelerate germination, reduce incidence of scabies (*Actinomyces scabies*), rhizoctonia (*Rhizoctonia solani*), alternaria (*Alternaria solani*) and fusarium (*Fusarium radici*), increase seed output, decrease seed output losses during long-term storage, etc. (Pavlista, 2011; Cheremisin and Kumpan, 2018; Araujo et al., 2019; Awati et al., 2016; Bhattarai, 2017; Bomok, 2019; Chekmarev et al., 2015; Głosek-Sobieraj et al., 2018; Gugała et al., 2019; Kumar at al., 2015; Lei, 2019; Naraghi et al., 2012; Otroshy and Struik, 2008; Pashkova and Kuz'minykh, 2018; Rex, 1992; Sekhon and Singh, 1985; Weiyan, 2015; Wierzbowska et al., 2015; Zamalieva et al., 2019).

There is currently no data on the effect of growth regulators Emistim C, Regoplant and

Stimpo on seed potato productivity in irrigated southern Ukraine. Therefore, there was a need to conduct research on the complex effect of fertilizers and growth regulators on the development and yield of seed potatoes of different ripeness groups.

The purpose of the research was to determine the yield, seed productivity and quality of biological ripeness potatoes under the action of growth regulators and different levels of mineral nutrition.

MATERIALS AND METHODS

Field experiments, laboratory and analytical studies during 2016-2018 were carried out at the Institute of Irrigated Agriculture of the NAAS of Ukraine (Kherson region, Naddniprians'ke) located on the right bank of the river Dnieper in the zone of the Ingulets irrigation system. The soil of the research plot is a dark chestnut medium-loamy. There was used the method of split plots with four repeats and two-row plots. The accounting area of the plot of the first order (variety factor) was 88.2 m^2, the second (factor of mineral nutrition level) - 29.4 m^2, the third (treatment with growth regulators) - 7.35 m^2, the total - 14.7 m^2. The area of nutrition of one plant was 70 × 25 cm. Previous crop was winter wheat (1st and 2nd year) and corn for grain. The agrotechnics used in the experiment were applied according to the guidelines for potato cultivation on irrigated land, developed by the Institute of Irrigated NAAS, except for the studied factors (Vozhehova et al., 2014b) and taking into account all the requirements of the method of research with potato (Kutsenko et al., 2002). The reliability of the results was determined using the Agrostat® software and information complex based on Microsoft Office® Excel®. The dry matter content was determined with gravity metod (GOST 13496.3-92); starch - for Evers, vitamin C - for Murri (GOST 24556-89); nitrates - potentiometric ion-selective electrode (GOST 13496.19-93).

We used as seed material the super-super elite of the varieties Skarbnytsya, Levada and Yavir breeding of the Institute of Potato NAAS, recommended for cultivation in the South of Ukraine in the experiment.

The Skarbnytsya is an early table, high-yielding variety. The taste qualities are good. Tubers are oval, yellow with creamy crumb. Levada is middle-early variety, tubers are rounded, light pink, creamy crumb. Yavir is a medium-ripe variety of tableware, high-yielding. Tubers are rounded, yellow, with a mesh skin, creamy crumb. These varieties are listed in the State Register of Plant Varieties Suitable for Distribution in Ukraine, respectively, from 2008, 2007 and 2000 (Institute of Potato and Institute of Irrigated Agriculture NAAS, 2012).

Mineral fertilizers in the form of nitroamophos (containing N, P and K content of 16%) were applied locally to the comb when planting at the rate of 45 or 90 kg of active substance nitrogen, phosphorus and potassium per hectare. The day before planting potatoes of the respective options were treated with 0.1% solution of Emistim C, Stimpo and 0.25% solution of Regoplant. In the phase of complete sprouting and budding, potato plants were treated with 0.01% solution of Emistim C, Stimpo and 0.025% solution of Regoplant. The growth regulators used in the experiment have a complex composition of plant origin regulators. Developer is "Agrobiotech".

Emistim C is a water-alcohol solution of metabolites of epiphytic fungi. It contains a complex of phytohormones of auxin, gibberellin and cytokinin nature, amino acids, carbohydrates, fatty acids, microelements. Regoplant is a multicomponent preparation for the products of vital activity of fungi-micromycetes from the root system of ginseng (saturated and unsaturated fatty acids - C14-C28), polysaccharides, 15 amino acids, analogues of the phytohormones of cytokinin and auxin nature) contains a complex of biogenic microelement, potassium salt of alpha-naphthylacetic acid and aversectin C. Stimpo includes the products of micromycetes fungi, a complex of biogenic microelement and aversectin C.

RESULTS AND DISCUSSIONS

The three-year average yield in the experiment is 18.86 t/ha. Variety characteristics did not affect on productivity - the average yield of the three varieties differed by a maximum of 0.21

t/ha, which was within the least significant difference for this factor (Table 1).

The average yield on non-fertilized variants was 14.68 t/ha. Application of $N_{45}P_{45}K_{45}$ increased the potato yield by 5.45 t/ha, or 37.1%, and the double fertilizer dose $N_{90}P_{90}K_{90}$ - by 7.09 t/ha (48.3%).

Table 1. Potato yield (t/ha) depending on variety, fertilizer and growth regulator, 2016-2018

Variety (factor A)	Fertilizer (factor B)	Growth regulator treatment (factor C)				Average by factors	
		Without treatment	Emistim C	Regoplant	Stimpo		
Skarbnitsa	without fertilizer	14.79	14.64	15.08	15.43	14.68	18.90
	$N_{45}P_{45}K_{45}$	19.51	20.22	21.85	21.12	20.13	
	$N_{90}P_{90}K_{90}$	21.79	21.49	20.28	20.59	21.76	
Levada	without fertilizer	14.88	14.43	14.33	14.20	18.75	18.94
	$N_{45}P_{45}K_{45}$	18.17	19.70	22.12	20.15		
	$N_{90}P_{90}K_{90}$	23.06	22.70	21.73	21.96		
Yavir	without fertilizer	15.45	14.29	14.01	14.64		18.73
	$N_{45}P_{45}K_{45}$	18.41	19.59	20.55	20.16		
	$N_{90}P_{90}K_{90}$	22.65	22.52	22.00	20.46		
Average by factors		18.75	18.84	19.11	18.75		
Significance of the main effects							
LSD_{05} A		LSD_{05} B				LSD_{05} C	
0.41		0.47				0.38	

Regulators influenced the yield - average yields in control without treatment and with Stimpo treatment were 18.75 t/ha, with treatment Emistim C - 18.84, Regoplant - 19.11; that is 0.1 and 0.36 t/ha (0.5 and 1.9%) more control.

Regulators combined with no fertilizer on all varieties except Skarbnitsa had a negative impact, reducing the yield from 1.0 to 9.3%. A double dose of fertilizers in combination with regulators also had some negative impact on the yield - from -0.6 to -9.7%. The decrease in potato yield on fertilizer-free and $N_{90}P_{90}K_{90}$ variants was due to the overdevelopment of the vegetative mass of potato due to the action of growth regulators.

The application of $N_{45}P_{45}K_{45}$ in combination with treatment with the investigated preparations had a positive effect overall, increasing the yield from 3.6 to 21.7%, depending on the variety and the regulator.

Maximum yield was 23.06 t/ha on variety Levada, without treatment, with $N_{90}P_{90}K_{90}$; the smallest was 14.01 t/ha on variety Yavir, without fertilizers and after treatment with Regoplant. The maximum positive effect of the growth regulator was recorded on the Levada variety with the $N_{45}P_{45}K_{45}$ and treated with Regoplant regulator - 21.7% of the control without treatment.

The highest yield of potato varieties was recorded on the $N_{90}P_{90}K_{90}$ variant without the use of growth regulators. This dose of fertilizer was recommended for potato cultivation under irrigation according to previous long-term studies (Vozhehova, 2014a). However, the yield of potato varieties with less than half the nutrition level and the use of growth regulators was almost at the recommended dose level. With this technology, material costs for mineral fertilizers are significantly reduced, as the cost of growth regulators is several times lower (7.5, 11 and 15 times).

The genotypic response to the use of stimulants was the highest in the Levada variety and the highest yield was obtained by treatment with Regoplant - 21.85 t/ha.

The smallest increase in yield due to the actions of regulators was observed in the Skarbnitsa variety - 0.71; 2.34 and 1.61 t/ha (3.6; 12.0 and 8.3%). In the Yavir variety, the increase was 1.18; 2.14 and 1.75 t/ha (6.4; 11.6; 9.5%). The best response to the treatment was received in the Levada variety - the increase was 8.4, 21.7 and 10.9%. The average increase from the treatment by regulators against the background of mineral fertilizer application at the dose of $N_{45}P_{45}K_{45}$ was 1.14, 2.81 and 1.78 t/ha (6.1; 15.0 and 9.5%) (Table 2).

Table 2. Effect of growth regulators on the background of $N_{45}P_{45}K_{45}$ on the yield and seed quality of potato, average for 2016-2018

Growth regulator	Yield, t/ha	Number of tubers per plant, pcs.	Weight of average tuber, g	Marketability, %	The output of the seed fraction, %	Weight of seed tubers, g
Without treatment	18.70	6.6	58.5	83.7	52.7	66.5
Emistim C	19.84	7.0	60.4	85.0	55.8	70.2
Regoplant	21.51	7.2	61.6	86.3	57.3	73.8
Stimpo	20.48	7.0	60.4	85.4	55.5	71.4

The maximum number of tubers from the plant in the experiment was formed by the variety Skarbnytsia - 7.6, Yavir slightly less - 6.9; least - Levada - 5.5 (Table 3). The application of $N_{45}P_{45}K_{45}$ and $N_{90}P_{90}K_{90}$ helped to increase the number of tubers by 1.2 and 1.5 pcs/bush; as a whole, only the treatment with the Regoplant regulator significantly increased the number of tubers, while on the background $N_{45}P_{45}K_{45}$ all stimulants increased the indicator by 0.4; 0.6 and 0.4 pcs.

As for the weight of average tuber, the effect of the variety is opposite - in the first place Levada - 71.7 g, in the second Yavir - 54.5 g; and Skarbnytsia on the latter - 49.8 g, which is 44% less than Levada. That is, the impact of the variety on this indicator, as on the previous one, is the most significant; after all fertilizers increased it by 6.6 g (12%) and 8.5 g (16%), and regulators as a whole are not reliable. Against the background of $N_{45}P_{45}K_{45}$ regulators significantly increased it by 1.9; 3.1 and 1.9 g (3; 5; 3%).

Table 3. Crop structure and seed properties of potatoes depending on variety, fertilizer and growth regulator (average by factors), 2016-2018

Variety/ Fertilizer/ Growth regulator	Number of tubers per plant, pcs	Weight of average tuber, g	Marketability, %	The output of the seed fraction, %	Weight of seed tubers, g
Skarbnytsia	7.6	49.8	82.0	56.2	61.0
Levada	5.5	71.7	88.1	51.5	75.1
Yavir	6.9	54.5	80.9	53.4	67.9
without fertilizer	5.8	53.6	79.8	48.8	59.3
$N_{45}P_{45}K_{45}$	7.0	60.2	85.1	55.3	70.5
$N_{90}P_{90}K_{90}$	7.3	62.1	86.1	56.9	74.3
without treatment	6.6	58.2	82.9	52.8	66.9
Emistim C	6.7	59.1	83.5	53.7	68.3
Regoplant	6.7	59.0	84.4	54.6	69.1
Stimpo	6.7	58.3	83.8	53.6	67.8
Significance of the main effects					
LSD_{05} variety	0.17	0.76	1.12	1,04	1.57
LSD_{05} / fertilizer	0.10	1.61	1.68	1,52	2.53
LSD_{05} growth regulator	0.14	1.35	1.33	1,62	2.13

The highest marketability is in the Levada variety (88%), the indicators of the variety Yavir and Skarbnytsya differed on 1% - 81-82%. Here the impact of the variety is also highest, but fertilizers at the same level increased the marketability of potatoes - by 5 and 6%; Regoplant - up to 1.5%. Against the background of the $N_{45}P_{45}K_{45}$, the percentage of Stimpo and Regoplant commercial potatoes was significantly increased by 1.6 and 2.5%, respectively.

For three years of research, the following indicators of seed productivity of potato varieties were established: Levada is characterized by the heaviest seed tuber among other varieties - 75.1 g and the lowest yield of the seed fraction - 51.5%. In the second place Yavir - 68 g and 53%, respectively. Skarbnytsya has the lightest seed tuber and the highest yield of this fraction - 61 g and 56%.

Fertilizers provided the following effect: 7% and 8% increased the yield of seed fraction and 11 and 15 g, respectively, of seed tubers (19% and 25%). On average, Regoplant increased by 2 g the weight of seed tubers and by 2% yield of the seed fraction (both indicators were at the level of the least significant difference). Against the background of $N_{45}P_{45}K_{45}$, the effect of all regulators was significantly higher: +4, +7, +5 g (6, 11, and 7%) of seed potato weight and +3, +5, +3% yield of seed fraction. The average yield of the seed fraction of potatoes was 10.26 t/ha. The lowest seed productivity was observed in the Levada variety - 9.85 t/ha, the highest in the Skarbnitsa variety - 10.76. The application of mineral fertilizers at a dose of $N_{45}P_{45}K_{45}$ increased the content of the conditioned seeds by 55.7%, $N_{90}P_{90}K_{90}$ by 72.1%, compared to the unfertilized control. The use of growth regulators did not affect the yield of the seed fraction of potatoes in the whole research. Against the background of the $N_{45}P_{45}K_{45}$, seed productivity increases were recorded for Stimpo (1.51 t/ha higher than the untreated control) and Regoplant (2.47 or 24.9% higher).

In 2016-2018, potatoes accumulated 21.7% of dry matter in tubers on average. In 2016, the average dry matter content was 21.9%, in 2017 - 23.4%; in 2018 - 19.7%.

Early-ripening Skarbnitsa and middle-ripe Yavir accumulated the same amount of dry matter - 22.5% (Figure 1).

The middle-early Levada is behind in this indicator by 19.9%. The application of mineral fertilizers increased its content by 0.9% and 1.1% in the three varieties. The use of growth regulators also had a positive impact on this indicator +0.8%; 1.1% and 0.8% of the whole research and +0.8; +1.5 and + 0.8% against the $N_{45}P_{45}K_{45}$ background.

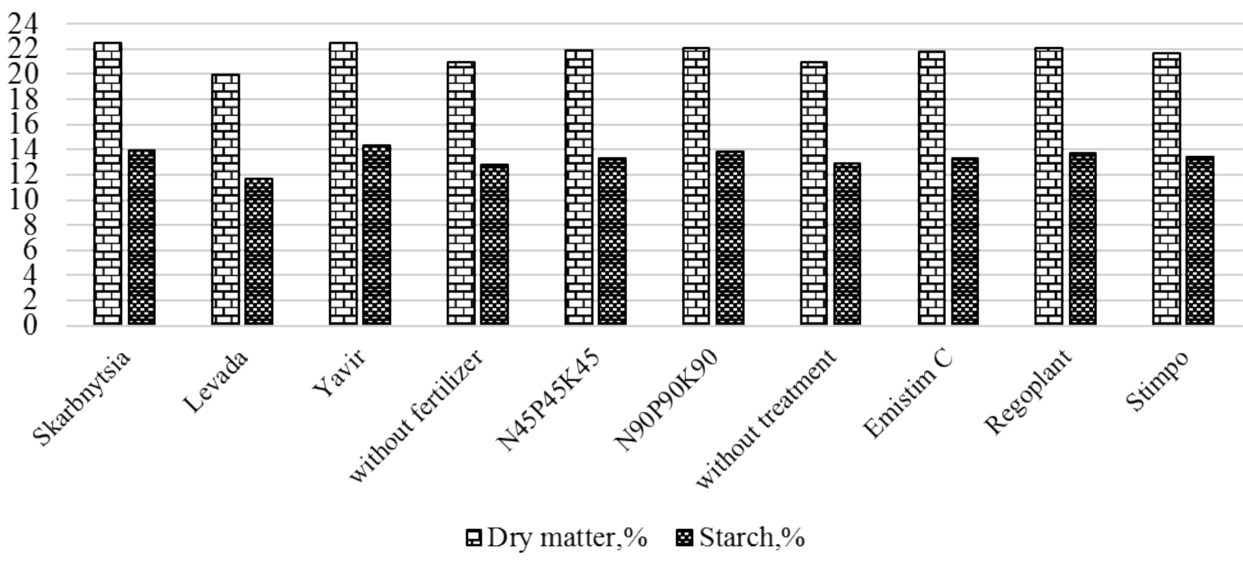

Figure 1. The content of dry matter and starch (%) in potatoes depending on the variety, fertilizer and growth regulator (average by factors), 2016-2018

The potato starch content in the three years averaged 13.3%. The relatively low starch content of the potato under study can be explained by the composition of the soil (on medium- and heavy-loam soils the starch content decreases) and the high temperatures during the period of reaching the tubers. Also, this indicator, as well as the previous one, differed significantly by years of research - 12.9% in 2016, 14.2% in 2017 and 12.7% in 2018. As we can see, 2017 was not only favorable for the formation of a high yield of potatoes (the highest in years of research), but also conducive for the accumulation of dry matter and starch in tubers. The most starchy variety was Yavir - 14.3%. The lowest starch content was in the Levada variety - 11.7%. The

Skarbnitsa took the intermediate place with 13.9% of starch.

Fertilizer application has influenced this indicator not so much as varietal features. Regulators increased potato starch content by 0.4, 0.8 and 0.5% at all nutrition levels and by 0.8, 1.4 and 0.9% against $N_{45}P_{45}K_{45}$. Vitamin C content was 173.5 mg/kg over three years. In 2016 - 206, in 2017 - 175, in 2018 - the least: 140 mg/kg. Varietal features influenced this indicator as follows: Skarbnitsa contained 178 mg, Yavir - 176, Levada - 167 mg/kg. Therefore, it can be concluded that, unlike the Skarbnitsa and Yavir varieties, Levada accumulates significantly less dry matter, starch and vitamin C.

There was a negative tendency of the effect of fertilizer on the accumulation of vitamin C: the application of $N_{45}P_{45}K_{45}$ reduced the vitamin C content by 6.9 mg/kg (3.9%); $N_{90}P_{90}K_{90}$ - by 13.5 mg/kg (7.5%). Regulators reduced vitamin C content, by whole, by 1.5; 2.9 and 3.2%. However, in most cases with $N_{45}P_{45}K_{45}$ there was even a slight increase over the untreated control. The content of nitrates in the tubers did not exceed the limit in Ukraine value (120 mg/kg) in all the years of the study. The average value is 57.5 mg/kg. In the Levada tubers it was 62.5 mg/kg, tubers of Skarbnitsa and Yavir accumulated 58.5 and 51.4 mg/kg nitrates respectively. On this indicator, the varietal characteristics were influenced not so much as the amount of fertilizer applied: tubers in variants without fertilizer accumulated 44.2 mg/kg nitrates, with $N_{45}P_{45}K_{45}$ - 57.46 mg/kg, with $N_{90}P_{90}K_{90}$ - 70.8 mg/kg. Thus, the application of these amount of fertilizer increased the nitrate content by 30.1 and 60.4%.

Regulators Emistim C and Stimpo reduced the average nitrate content by 2.0 and 5.5%, respectively. For this indicator there is a noticeable difference of influence of regulators depending on the level of mineral nutrition: on the background without fertilizers there is an increase of nitrate content from treatment by regulators from 1.9 to 12.0%; on the $N_{45}P_{45}K_{45}$ background, regulators also increase nitrate content (except Stimpo); while on a high fertilizer background, nitrate content is reduced to 21.3%. In the experiment of Pavlista (2011) also used growth regulators of complex

Application of $N_{45}P_{45}K_{45}$ increased starch content from 12.8 (control variants) to 13.3% (by 0.5%); $N_{90}P_{90}K_{90}$ - up to 13.7% (0.9%).

composition, which at early harvest increased the yield of the Atlantic potato by 13-15%. In the experiment of Chekmarev et al. (2015), a two-fold treatment with commercial growth regulators increased yields by 5.18-7.16 and 4.16-5.20 t/ha. Głosek-Sobieraj et al. (2018) have shown that complex growth regulators increase the yield and the percentage of medium-sized tubers.

In the experiment of Awati et al. (2016), growth regulators increased the total yield, the number of tubers from the bush from 2.6 to 3.4 pcs. In the Bhattarai (2017) experiment, paclobutrazole treatment increased the weight and size of tubers. The use of ethephon growth regulators (ETH) and chlormequat chloride (CCC) in different doses has led to an increase the total number of tubers and a decrease in the weight of tubers, as a consequence - a decrease in marketability (Sekhon and Singh, 1985). The yield of marketable potatoes from the combined use of growth stimulant at the beginning of the growing season and the inhibitor at the end of the growing season increased by 9.16% in the experiment of Kumar et al. (2015). With the use of Epin Extra Pashkova and Kuz'minykh (2018), the crop increased by 1.9 t/ha, the dry matter content increased by 2.3-2.6% and the starch by 0.7-1.3%. In the experiment of Araujo et al. (2019), potato were treated with paclobutrazole (PBZ) and trinexapac-ethyl (TE) at the end of the growing season, which led to a decrease in starch and reduced sugars content in tubers. In addition to the growth regulator and the weight of the seed potatoes, the potato growth parameters were significantly influenced by the variety factor (Otroshy and Struik, 2008).

Wierzbowska et al. (2015) stated that although biostimulants improved the quality of potatoes in their experiment, it was more dependent on the characteristics of the variety than on the biostimulants used.

CONCLUSIONS

Maximum productivity of early and middle-early varieties Skarbnitsa and Levada was formed by the application of mineral fertilizers at a dose of $N_{45}P_{45}K_{45}$ treated with growth regulator Regoplant - 21.85 and 22.12 t/ha. The middle-ripe variety Yavir is better grown with $N_{90}P_{90}K_{90}$, with a yield of 22.65 t/ha. The genotypic response to stimulant use was highest in the Levada variety. The growth regulators increased yield of tubers biological maturity on the background $N_{45}P_{45}K_{45}$ by 1.14 t/ha (Emistim C); 1.78 t/ha (Stimpo) and 2.81 t/ha (Regplant) compared to untreated control. The number of tubers, the weight of average tuber, the amount of dry matter and starch, the content of nitrates were influenced by fertilizers and growth regulators, although most significantly - the characteristics of studied varieties. Fertilizer application had a negative impact on vitamin C.

The application of mineral fertilizers at a dose of $N_{45}P_{45}K_{45}$ and the complex treatment of tubers and plants with growth regulators have increased the seed productivity of potato varieties. The output of the seed fraction increased by 7% (action of fertilizers) and 3-5% (action of regulators), the mass of seed tubers - by 19 and 6-11%. The highest profitability in the experiment - against the background of $N_{45}P_{45}K_{45}$ with Levada cultivar treatment - 149.2%. With the $N_{45}P_{45}K_{45}$, regulators increased profitability of ssed popato production by 10.1% (Emistim C), 15.8% (Stimpo) and 24.7% (Regoplant).

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THERMO-MECHANICAL AND BREADMAKING PROPERTIES OF WHEAT-SORGHUM FLOUR BLENDS

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Abstract

Sorghum flour is a valuable ingredient for breadmaking. The thermo-mechanical properties of dough prepared out of wheat flour supplemented with 10, 20, 30, 40 and 50% sorghum flour and the bread characteristics in terms of specific volume, crumb firmness, resistant starch, total phenols and antioxidant activity were investigated. Water absorption and dough stability decreased when increasing the level of sorghum flour as a consequence of the dilution effect of gluten. Starch gelatinization speed was 0.50 Nm/min in case of wheat flour and increased up to 0.70 Nm/min for sample with 50% sorghum flour. In addition, gel stability during heating increased with the level of sorghum flour. Although wheat flour substitution by sorghum flour affected the specific volume of the bread and crumb firmness, the level of biologically active compounds in the final products was significantly increased. In particular, the addition of 50% sorghum flour caused the increase of the total phenols content and DPPH-RSA of bread from 122.41 to 527.59 μmol FAE/g d.w., and from 3.90 to 25.59%, respectively. Moreover, the content of resistant starch of the breads increased with the level of sorghum flour, most probably as a consequence of the presence of polyphenols from sorghum flour and of the interaction between sorghum starch and proteins.

Key words: *wheat, sorghum, thermo-mechanical properties, bread, resistant starch.*

INTRODUCTION

Sorghum has a major role in sustainable grain production and an increasing importance to food security (Khan et al., 2013). It is a cereal with wide adaptation to the environmental conditions, to the abiotic stress tolerance, having good adaptation on the increase of temperatures and decrease of precipitation, and low requirements of fertilization and crop protection products (Berenji and Dahlberg, 2004).

In recent years several studies highlighted the nutritional potential of sorghum. Sorghum contain a large spectrum of phenolic compounds, the most important being phenolic acids and flavonoids (Taylor et al., 2014). The types and levels of the phenolic compounds depend by the sorghum variety: white variety contains especially simple phenolic acids, while red and brown varieties contain phenolic acids, anthocyanins and condensed tannins. Additionally, sorghum is a source of other functional ingredients such as resistant starch (Khan et al., 2013; Yousif et al., 2012). Due to all these components sorghum is considered a cereal with valuable health promoting potential, being used as ingredient in baked products based on either wheat or non-wheat flours, including gluten free flours (Khan et al., 2013; Yousif et al., 2012; Onyango et al., 2009; Onyango et al., 2011; Vasquez et al., 2016; Rai et al., 2014; Ferreira et al., 2016).

There are certain properties of some components, such as proteins and starch, which might limit the use of sorghum for obtaining baked products (Onyango et al., 2011). Taylor et al. (2006) identified the main problems that limit sorghum applications in breadmaking, namely the lack of viscoelastic protein network and quick staling. According to Duodu et al. (2002) and Onyango et al. (2011), sorghum proteins form aggregates during cooking. Schober et al. (2007) reported that sorghum proteins found in the batter are prone to strands and lumps formation due to aggregation, which interfere with the starch gel during baking, therefore resulting in flat breads with holes in the crumb. These final products usually have poor capacity to retain the fermentation gases produced during proofing and in the early stages of baking. Sorghum starch has high gelatinization temperature that can lead to a poor gelatinization during baking, with negative impact on sensorial properties of the final products (Torbica et al., 2019; Onyango et al., 2011).

The aim of the presents study was to investigate the effect of the wheat flour supplementation with sorghum flour on the thermo-mechanical properties of dough and on the physical-chemical characteristics of the bread. In particular, the presence of resistant starch, the content of total phenols and antioxidant activity of the final products prepared with different amounts of sorghum flour were tested.

MATERIALS AND METHODS

Materials

The commercial white wheat flour (WF) (Boromir, Romania) and sorghum whole flour (SF) (origin Hungary, distributed by Adams Vision SRL, Târgu Mureș, Romania) were purchased from the local market (Galați, Romania).

Proximate analyses

In order to establish the proximate composition of the flour samples, the following standard methods were used: SR ISO 712:2005 (ASRO, 2008) for moisture, semimicro-Kjeldahl method (Raypa Trade, R Espinar, SL, Barcelona, Spain) for protein (nitrogen conversion factor of 5.75 for wheat flour, and 5.81 for sorghum flour), Soxhlet extraction method with ether (SER-148; VELP Scientifica, Usmate Velate (MB), Italy) for fat, Fibretherm Analyser based method (C. Gerhardt GmbH & Co. KG, Germany) for crude fiber, and SR ISO 2171/2002 (ASRO, 2008) for ash. The starch content was established by subtracting the total percentage of the assayed chemical components from one hundred.

Amylose content was determined using Amylose/Amylopectin assay kit (Megazyne International Ireland Ltd. Wicklow, Ireland), following the manufacturer's procedure based on the methods of Gibson et al. (1997).

Starch damage was determined by spectrophotometric method (AACC Method 76-31.01) using dedicated assay kit from Megazyne International (Ireland Ltd. Wicklow, Ireland).

Physical and functional properties

Fineness modules were determined according to Godon and Willm (1994) method, using in sequence sieves with 400, 315, 160 and 125 μm mesh.

The brightness value L*, redness value a* and yellowness value b* were measured by means of Chroma Meter CR-410 (Konica Minolta Business Solutions Europe GmbH).

Solvent retention capacity (SRC) test was performed according to AACC Method 56-11.02, by independently testing the retention of the following solvents: water (W-SRC), 5% lactic acid (LA-SRC), 5% sodium carbonate (SC-SRC) and 50% sucrose (S-SRC).

The method proposed by Abebe et al. (2015) and slight modified by Villanueva et al. (2018) was employed for determining the water absorption index (WAI), water solubility index (WSI), and swelling power (SP). The flour samples (2.5 g) were mixed with 30 ml distilled water in pre-weighed centrifuges tubes, cooked at 30 and 90°C in water bath for 10 min, cooled down to room temperature and further centrifuged at 4,000 x g for 10 min. The WAI was determined as ratio between the weights of the sediment resulted after centrifugation, and of the flour sample. The soluble solid of the supernatants was measured after evaporation overnight at 110ºC. The WSI was determined as ratio between soluble solid of the supernatant, and the weight of the flour sample. The SP was calculated by dividing the soluble solid of the supernatant and by the difference between weight of flour sample and soluble solid resulted after evaporating.

Thermo-mechanical properties

The thermo-mechanical properties of white wheat flour (control), sorghum flour and mixtures of different ratios of wheat flour: sorghum flour (90: 10, 80: 20, 70: 30, 60: 40, 50: 50) were determined using the Mixolab device (Chopin Technology, Villeneuve La Garenne, France). The Chopin+ protocol was applied for all wheat flour based samples, while for the sorghum whole flour the Chopin+ protocol was adapted by modifying the dough weight to 90 g instead of 75 g. The following thermo-mechanical parameters were registered from the typical Mixolab curves: C2, C3, C4 and C5 torques related to protein weakening while mixing and heating, starch gelatinization, hot gel stability and retrogradation in the cooling phase, respectively.

The cooking stability and breakdown were additionally estimated through the (C4/C3) and (C3-C4), while protein weakening speed during heating, starch gelatinization speed and enzymes degradation speed, through the alpha, beta and gamma slopes of Mixolab curve, respectively (Dubat and Boinot, 2012; Svec and Hruskova, 2015).

The bread-making procedure

The white wheat flour was blended with sorghum whole flour in the ratios of 100: 0, 90: 10, 80: 20, 70: 30, 60: 40, 50: 50.

The doughs were prepared through the one stage method using the following formula on the 100 g mixture of white wheat flour and sorghum whole flour: 1.5% salt, 3% compressed baker's yeast, and water (according to the water absorption capacity established through Mixolab tests for each flour mixture). The bread-making procedure is described in Banu et al. (2010).

Bread analysis

Specific volume and crumb firmness

The specific volume of the bread and crumb firmness were determined after storing the samples for one hour at room temperature. The specific volume was determined using SR 91/2007 method (ASRO, 2008) based on rapeseed displacement.

The MLFTA apparatus (Guss, Strand, South Africa) and a probe with diameter of 7.9 mm were used to measure crumb firmness on two bread slices from the center of every sample. The following parameters were used for measuring crumb firmness: penetration wide of 25 mm, test speed of 5 mm/s, and trigger threshold force of 1.96 N (Banu et al., 2017).

Resistant starch

Resistant starch was determined using the Megazyne assay kit (Megazyne International Ireland Ltd. Wicklow, Ireland), according to the manufacturer's procedure based on the AACC Method 32-40.01.

Total phenol and antioxidant activity

In order to determine the total phenolic contents (TPC) and DPPH-radical scavenging activity (DPPH RSA) of bread an extraction procedure with 80: 20 methanol/water (v/v) solvent mixtures was used. The total phenolic contents (TPC) were determined following the Folin-Ciocalteu method proposed by Singleton and Rossi (1965) and modified by Gao et al. (2002). The results were expressed as mg ferulic acid equivalent (FAE) per g d.w., ferulic acid being used as standard. DPPH-radical scavenging activity was measured using the method described by Brand-Williams et al. (1995) and modified by Beta et al. (2005).

Statistical analysis

Triplicate experiments were carried out in, and the results are reported as average values together with standard deviation. Analysis of variance, performed with Minitab 18 Statistical Software, was used to check any significant differences among samples with different percentages of sorghum flour. The normality and variance equality conditions were initially check and differences were afterwards quantified using one-way ANOVA with a 95% confidence interval.

RESULTS AND DISCUSSIONS

Proximate composition of flours

The proximate composition of sorghum and wheat flours is presented in Table 1. When compared to the wheat flour, sorghum flour had higher contents of fat (3.17%), crude fiber (3.43%), and ash (1.61%). According to Vargas-Solorzano et al. (2014), the composition of sorghum flour is related to the genotype of the grain – white, red and brown sorghum. The authors noted that the flour obtained from brown sorghum had higher fat, ash and fiber, compared to the flour resulted from red and white sorghum. The brown sorghum flour can get 3.32-3.18% fat, 1.59-2.47% ash, and 12.86-11.58% neutral detergent fiber, as against 1.73-1.76% ash, 2.96-3.05% fat, and 8.52-8.93% neutral detergent fiber in case of red and white sorghum flour. Rai et al. (2014) reported for the whole sorghum flour the following composition: 4% fat, 2.2% ash, 2.30% crude fiber, and 72.2% starch. The amylose content of the flour has a major role in starch digestibility and in further formation of resistant starch (Yousif et al., 2012). As indicated in Table 1, the amylose content of sorghum and wheat flours was 23.03 and 27.57%, respectively. Our results are in agreement with Yousif et al. (2012), who reported amylose contents of 24.7 and 27.6%, in case of white and red sorghum flours, respectively, while other studies quoted by these authors indicated amylose contents ranging from 24 to 33%.

Regarding starch damage, the results presented in Table 1 revealed higher value (8.17%) for sorghum flour, compared to the wheat flour (5.13%). However, Yousif et al. (2012) reported higher values for starch damage of white sorghum flour and red sorghum flour, of 12.03 and 9.43%, respectively.

Table 1. Proximate composition of the sorghum and wheat flours

Component	Sorghum flour	Wheat flour
Moisture (%)	10.29±0.02	12.30±0.02
Protein (% d.w.)	7.88±0.07	9.29±0.04
Fat (% d.w.)	3.17±0.07	1.04±0.03
Crude fiber (% d.w.)	3.43±0.04	2.28±0.03
Ash (% d.w.)	1.61±0.01	0.48±0.01
Starch (% d.w.)	73.57±0.07	74.65±0.04
Amylose (% d.w.)	23.03±0.15	27.57±0.43
Damage starch (% d.w.)	8.17±0.15	5.13±0.15

Physical and functional properties of flours

The wheat flour had lower fineness module (1.13), compared to the sorghum flour (1.53) (Table 2). Sorghum flour contained a higher percentage (~ 62%) of large particles, with size ranging from 160 to 315 μm, while in the wheat flour prevailed the particles having size between 125 and 160 μm (68.5%). The particles size distribution, along with chemical composition of flours, influenced the hydration properties. If in case of wheat flour the presence of appropriate amounts of water is necessary for developing the gluten network, in case of gluten free flour water is necessary to hydrate the flour constituents. Moreover, Collar and Angioloni (2014) appreciated that hydration properties pays a key role in modulating dough workability and the properties of the end-products.

Solvent retention capacity, measured using as solvents water, lactic acid, sucrose and sodium carbonate, represents a useful method which allows describing the functional profile of the flour. It is considered that a well performing system of sponge and dough should have the following solvent retention capacity profile: W-SRC ≤ 57%, S-SRC ≤ 96%, LA-SRC ≥ 100%, SC-SRC ≤ 72% (AACC method 56-11.02). The results presented in Table 2 revealed important differences between SRC profiles of sorghum and wheat flour. The higher levels of fiber and starch damage of sorghum flour resulted in higher values of S-SRC, SC-SRC and W-SRC. The LA-SRC value is quite large due to the kafirins and glutelins from sorghum flour composition. The SRC profile of sorghum flour is quite similar to SRC profile to teff flour reported by Collar and Angioloni (2014): W-SRC of 111%, S-SRC of 145%, LA-SRC of 129% and SC-SRC of 120%. As expected, the gluten performance index (GPI), which is a measure of the overall performance of glutenin in the complex network (Kweon et al., 2011), was lower for sorghum flour (0.48) than wheat flour (0.69). Mariotti et al. (2016) reported lower GPI values of 0.439 and 0.527 for commercial gluten free flours, compared to the hard wheat flours (GPI of 0.628).

The color parameters of flours are depicted in Table 2. The lightness value (L*) of wheat flour was high, over 93, being specific to a white flour, while the L* value of sorghum flour was much lower, about 82, due the high content of tannins and phenolic compounds from sorghum (Vargas-Solorzano et al., 2014). Similar yellowness values were measured for both investigated flours, while the a* values varied from redness (+4.50) for sorghum flour to greenness (-1.28) for wheat flour (Table 2).

The hydration properties, appreciated in terms of WSI, WAI and SP, increased with the temperature increase from 30 to 90°C. The most significant increase was noticed for the wheat flour. The lower values of WSI registered for sorghum flour can be explained by the complexed formed between starch and kafirins (Chandrashekar and Kirleis, 1988) or lipids (Kraithong et al., 2018), the latter being in higher amount in sorghum flour compared to the wheat flour. Chandrashekar and Kirleis (1988) suggested that, because of the high content of kafirin, sorghum is prone to higher gel consistency and lower degree of starch gelatinization. It was suggested that kafirin might organize as a barrier around the starch granule, limiting starch gelatinization. The authors noted that the enthalpy of dissociation of amylose-lipid complexes is higher in case of sorghum than in millet, but is lower compared to oat. The same complexes formed between starch and kafirin and lipids seem to have the main influence on WAI and SP value.

Table 2. Physical and functional properties of sorghum and wheat flours

Component		Sorghum flour	Wheat flour
Physical properties			
Fineness modules		1.53±0.03	1.13±0.03
Color values	L*	81.92±0.30	93.16±0.04
	a*	4.50±0.01	-1.28±0.01
	b*	12.49±0.04	12.38±0.05
Solvent retention capacity			
Water, %		117.50±3.52	73.30±3.25
Sucrose, %		149.30±2.26	95.70±2.73
Sodium carbonate, %		121.30±2.28	87.20±2.17
Lactic acid, %		130.40±3.36	126.90±2.24
GPI		0.48±0.00	0.69±0.01
Hydration properties			
Water solubility index, %	30°C	2.16±0.05	1.79±0.08
	90°C	4.28±0.07	6.43±0.04
Water absorption index, g/g	30°C	4.29±0.05	4.58±0.08
	90°C	4.83±0.05	10.11±0.10
Swelling power, %	30°C	4.48±0.08	4.80±0.05
	90°C	5.08±0.07	11.25±0.09

Thermo-mechanical properties of dough prepared from wheat flour supplemented with sorghum flour

The parameters describing the thermo-mechanical behavior of the dough prepared from wheat flour supplemented with different percentages of sorghum flour are shown in Table 3. Additionally, the Mixolab curves are depicted in Figure 1. Water absorption of wheat flour was 56.8% and gradually decreases to 55.4% with the increase of the sorghum flour level. This trend might be explained by the differences in terms of water absorption capacity of proteins from wheat and sorghum. The gluten proteins from wheat are able to bind higher amounts of water, while kafirins, the major proteins from sorghum, are more hydrophobic and bind lower amount of water (Yousif et al., 2012; Belton et al., 2006). However, the difference between WA of control and sample with 50% sorghum flour addition is not very large, most probably due the contribution of starch damage to the WA; higher starch damage was obtained in case of sorghum flour compared to the wheat flour (Table 1).

Dough stability decrease from 9.50 min for wheat flour to 4.90 min for wheat sample supplemented with 50% sorghum flour, due to the gluten dilution effect, coupled with gluten network disruption by chemicals components from sorghum flour. Yousif et al. (2012) reported that the levels of dough stability decrease depend by the type of sorghum flour incorporated into the wheat flour. Thus, in case of using white sorghum flour, dough stability decrease from 12.7 min to 9 min with increasing the levels of sorghum flour addition from 0 to 50%, while the same levels of red sorghum flour addition resulted in dough stability decrease to 5.8 min.

The C2 values slightly increased with the addition level of sorghum flour (Table 3), and this resistance of dough against deformation during kneading and heating is most likely due to the fiber content and fiber compositions. According to Vargas-Solorzano et al. (2014), the sorghum arabinoxilans are highly substituted and contain uronic acids, acetyl and feruloyl substituents, which result in a matrix which lacks flexibility (Nandini and Salimath, 2001). The C3 was in general reduced when increasing the level of sorghum flour; only a slight increase was observed for dough samples with 10 and 20% sorghum flour (Table 3). The starch gelatinization temperature decreased with increasing the wheat flour substitution level by sorghum flour. Torbica et al. (2019) and Onyango et al. (2011) indicated gelatinization temperature of 71-72°C for sorghum flour, lower compared to the wheat flour. Our results indicated that sorghum flour addition caused the significant decrease of the temperature corresponding to the C3 torque, from 81.6°C, obtained in case of wheat flour, to 77.4°C, for sample with 50% sorghum flour (Table 3). Starch gelatinization speed (beta slope) increased from 0.50 Nm/min, for wheat flour, to 0.70 Nm/min for dough sample with 50% sorghum flour. Hot gel stability (C4), cooking stability (C4/C34) and retrogradation in the cooling phase (C5) followed the same trend (Table 3). Different trends were registered between the C3 and C4 torques in the Mixolab curves of dough samples with increasing levels of sorghum flour; wheat flour substitution resulted in the increase torque values, thus the breakdown (C3-C4) registered negative values. This suggests a higher stability of gel during heating due to the decrease of amylase activity through wheat substitution by sorghum flour.

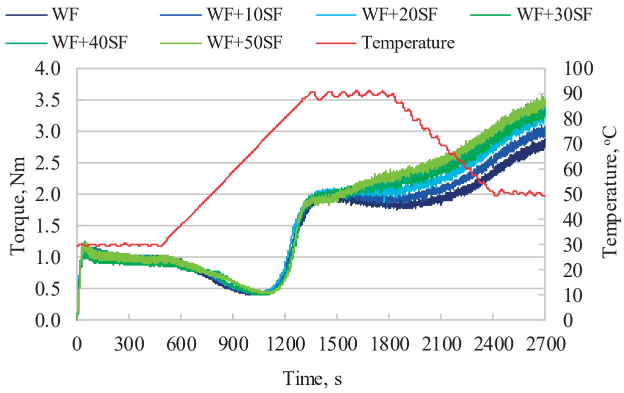

Figure 1. The Mixolab curves recorded for the blends consisting of wheat flour (WF) supplemented with different percentages (10-50%) of sorghum flour (SF)

Bread analysis

Table 4 shows the physical properties of bread samples in terms of specific volume, crumb firmness and color of bread crumb. The specific volume of the bread samples decreased with the level of sorghum flour. This decrease can be mainly attributed to the gluten dilution effect. Additionally, Taylor et al. (2006) suggested that responsible for lower volume of the bread prepared with sorghum flour might be also lipid properties, more precisely the lack of glyco and phospholipids in sorghum, when compared to the wheat. The properties of lipids as well as those of starch influence the crumb firmness. Thus, crumb firmness increased with the level of sorghum flour in the bread formulation (Table 4). The lightness value (L*) presented a significant decrease from 73.32, corresponding to the wheat flour bread, to 54.64 measured on bread sample with 50% sorghum flour. A decreasing trend was observed also in the yellowness values (b*), but to a lesser extent compared to L*. Regarding a*, all values were positive, suggesting that the red tone is dominant, most probably due the presence of high levels of anthocyanins (Yousif et al., 2012).

Table 5 shows the results of total phenol contents, DPPH-radical scavenging activity and resistant starch of bread samples. When increasing the wheat flour substitution level by sorghum flour from 0 to 50%, the TPC and DPPH-RSA of bread samples increased from 122.41 to 527.59 µmol FAE/g d.w., and from 3.90 to 25.59%, respectively. These results are explained through the TPC of wheat and sorghum flours used for preparing the breads. Thus, TPC and DPPH-RSA of wheat flour were 208.62 µmol FAE/g d.w. and 5.99%, respectively, while in case of sorghum flour, the TPC and DPPH-RSA were 1717.24 µmol FAE/g d.w. and 68.77%, respectively. According to Dlamini et al. (2007) the total phenolic content depends by the type of sorghum. Thus, the sorghum with pigmented testa, that has higher tannin content, had higher levels of TPC. Additionally, these types of sorghum had higher antioxidant activity. The phenolic acids are spread in different parts of the kernel: the free phenolic acids, represented by ferulic, p-cumaric, vanilic, caffeic, p-hydroxybenzoic and protocatechuic, are concentrated in the outer layers, namely pericarp, testa, and aleurone, while the bound form of phenolic acids, represented by gallic and cinnamic acids, are mainly associated with the cell walls (Dykes and Rooney, 2006).

The resistant starch of the bread samples increased with the level of sorghum flour used to substitute the wheat flour. Austin et al. (2012) and Taylor and Emmambux (2010), quoted by Khan et al. (2013), suggested that the inhibitory effect exerted by the sorghum polyphenols on digestive enzyme activity and the particularities of the interactions between starch and proteins from sorghum are mainly responsible for the higher content of resistant starch found in case of sorghum flours compared to the durum wheat semolina. Khan et al. (2013) reported the increase of the resistant starch of pasta prepared with wheat flour semolina and different levels of sorghum flour. Moreover, the authors noted that when red sorghum flour was used, the resistant starch from pasta was higher compared to the corresponding white sorghum flour based samples.

Table 3. Thermo-mechanical properties of the dough samples based on wheat flour (WF) supplemented with different percentages (10-50%) of sorghum flour (SF)

Parameters	Samples					
	WF	WF+10SF	WF+20SF	WF+30SF	WF+40SF	WF+50SF
WA, %	56.80±0.13[a]	56.00±0.13[b]	55.80±0.10[b,c]	55.60±0.10[c,d]	55.60±0.10[c,d]	55.40±0.13[d]
S, min	9.50±0.13[a]	8.70±0.13[b]	8.00±0.13[c]	7.50±0.13[d]	5.20±0.09[e]	4.90±0.13[e]
C2, Nm	0.42±0.02[b]	0.42±0.01[a,b]	0.43±0.01[a,b]	0.43±0.01[a,b]	0.44±0.01[a,b]	0.44±0.01[a]
C3, Nm	1.95±0.01[c]	2.01±0.00[a]	1.99±0.01[b]	1.94±0.01[c]	1.93±0.01[c]	1.91±0.01[d]
TC3°C	81.60±0.17[a]	80.90±0.17[b]	79.90±0.10[c]	77.60±0.10[d]	75.60±0.17[e]	77.40±0.10[d]
C4, NM	1.82±0.01[f]	1.91±0.02[e]	2.06±0.01[d]	2.16±0.01[c]	2.21±0.01[b]	2.27±0.01[a]
C5, Nm	2.80±0.01[e]	3.01±0.01[d]	3.24±0.01[c]	3.30±0.01[b]	3.44±0.01[a]	3.45±0.01[a]
C3-C4, Nm	0.14±0.00[a]	0.10±0.02[a]	-0.07±0.02[b]	-0.22±0.02[c]	-0.27±0.02[d]	-0.36±0.00[e]
C3/C4	0.93±0.00[f]	0.95±0.01[e]	1.04±0.01[d]	1.12±0.01[c]	1.14±0.01[b]	1.19±0.01[a]
Alpha, Nm/min	-0.09±0.01[c]	-0.10±0.01[c]	-0.11±0.01[c]	-0.09±0.01[c]	-0.01±0.01[a]	-0.05±0.01[b]
Beta, Nm/min	0.50±0.01[e]	0.66±0.01[b]	0.55±0.01[d]	0.53±0.01[d]	0.59±0.01[c]	0.70±0.01[a]
Gamma, Nm/min	-0.02±0.01[d]	-0.02±0.01[d]	0.020.01[c]	0.06±0.01[b]	0.06±0.01[b]	0.08±0.01[a]

The mean values in each line that do not share a letter are statistically significant ($p \leq 0.05$).

Table 4. Physical properties of bread samples prepared with blends consisting of wheat flour (WF) supplemented with different percentages (10-50%) of sorghum flour (SF)

Breads	Specific volume, cm^3/g	Crumb firmness, g force	Color of bread crumb		
			L*	a*	b*
WF	3.66 ±0.10[a]	763.05±3.69[f]	73.32±0.14[a]	0.40±0.05[f]	24.78±0.11[a]
WF+10SF	3.45±0.08[b]	1045.00±2.65[e]	67.37±0.10[b]	2.38±0.08[e]	19.91±0.09[b]
WF+20SF	3.28±0.05[b]	1086.41±2.12[d]	63.07±0.12[c]	4.00±0.05[d]	19.33±0.04[c]
WF+30SF	2.87±0.05[c]	1154.22±3.01[c]	59.05±0.09[d]	5.03±0.05[c]	18.54±0.07[d]
WF+40SF	2.76±0.05[c]	1321.24±2.39[b]	56.67±0.12[e]	6.27±0.10[b]	18.68±0.09[d]
WF+50SF	2.41±0.08[d]	1351.94±2.25[a]	54.64±0.07[f]	6.79±0.12[a]	17.82±0.03[e]

The mean values in each column that do not share a letter are statistically significant ($p \leq 0.05$).

Table 5. Total phenol, antioxidant activity and resistant starch of bread samples prepared with blends consisting of wheat flour (WF) supplemented with different percentages (10-50%) of sorghum flour (SF)

Breads	Total phenol content, µmol ferulic acid equiv/g d.w.	DPPH-radical scavenging activity, %	Resistant starch, %
WF	122.41±0.72[f]	3.90±0.10[f]	1.09±0.10[f]
WF+10SF	156.90±0.45[e]	8.47±0.16[e]	1.40±0.11[e]
WF+20SF	329.31±0.54[d]	12.98±0.21[d]	2.04±0.08[d]
WF+30SF	372.41±0.72[c]	15.93±0.18[c]	2.63±0.10[c]
WF+40SF	450.00±0.72[b]	19.60±0.15[b]	3.04±0.09[b]
WF+50SF	527.59±0.76[a]	25.59±0.23[a]	3.45±0.08[a]

The mean values in each column that do not share a letter are statistically significant ($p \leq 0.05$).

CONCLUSIONS

Thermo-mechanical properties of dough are modified with increasing the substitution level of wheat flour by sorghum flour. Water absorption and dough stability decreases due the dilution effect of gluten coupled with gluten network disruption caused by different compounds arising from sorghum flour. The protein weakening slightly increased with the addition level of sorghum flour. The dough resistance to deformation during kneading and heating was mainly ascribed to the presence and profile of fibers in the complex dough matrix. Starch gelatinization speed, hot stability and cooking stability increased with levels of wheat flour substituted with sorghum flour. The baking test indicated that the specific volume of the wheat flour based bread samples decreased, while the crumb firmness increased with the level of sorghum flour in the mixture. The red tone was found to be dominant in the breads crumb and was ascribed to the presence of anthocyanins. Finally, the addition of sorghum flour to the wheat flour resulted in bread samples with increased total phenols content, antioxidant activity and resistant starch.

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THE USE OF ALFAXALONE IN COMBINATION WITH OPIOIDS FOR CAT SEDATION: PRELIMINARY RESULTS

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Abstract

This study describes a prospective, randomized, blinded clinical study on 118 cats which required chemical restraint. The protocol of sedation was based on a combination of alfaxalone and an opioid among methadone, butorphanol and pethidine in order to improve the quality of sedation, offset the lack of analgesia and reduce side effects of alfaxalone. The patients underwent clinical evaluation and each of them was randomly allocated to one of the 3 groups. The evaluation procedure lasted 30 minutes from sedation, during which cats were monitored to fill an evaluation sheet at predefined time points. The quality of recovery was influenced by the opioid, with a higher score for butorphanol and pethidine, but also by body condition score (BCS) and age. The time of lateral recumbency was affected by the American Society of Anesthesiologists score (ASA), and it was higher in ASA 1-2. Physiological parameters were influenced by the molecule over time. The molecules influenced also the degree of muscular relaxation and quality of sedation. The last parameter was also influenced by ASA status with a greater score in cats classified as ASA 3.

Key words: *alfaxalone, cat, opioid, sedation.*

INTRODUCTION

Cats are notoriously sensitive animals, whose emotionality can be upset in a veterinary environment causing anorexia, abnormal behaviour, change of physiological parameters and immunosuppression. For these reasons veterinarians often recur to chemical restraint (Pascal et al., 2013; 2015; Neagu et al., 2018).

Drugs such as ketamine and α_2-agonists represent the first choice in clinical practice, although they have an important impact on main systems of the organism, resulting not suitable in patients with head trauma, cardiac and respiratory disease, liver and kidney failure.

The present work describes a prospective, randomized, blinded clinical study on 118 cats in order to validate a safe and efficacy protocol of sedation using alfaxalone in combination with three different opioids among methadone, butorphanol and pethidine.

The aims of the study are the following: improvement of quality of sedation, finding an alternative to common protocols which use ketamine and α_2-agonist, offset the lack of analgesia and reduce side effects of alfaxalone such as tremors, hyperextension of neck and limb, opisthotonus, uncontrolled movements, maintaining an optimal cardiovascular and respiratory stability.

MATERIALS AND METHODS

One hundred and eighteen cats that needed sedation, presented at the Veterinary Teaching Hospital of the University of Perugia, were recruited for this study. The patients underwent a clinical evaluation: physiological parameters were measured, ASA status was identified and each of them was randomly allocated to one of three groups identified. The protocol of sedation was based on a combination of 3 mg/kg alfaxalone and an opioid among 0.3 mg/kg methadone (M group), 0.3 mg/kg butorphanol (B group) and 5 mg/kg pethidine (P group) administered IM.

The procedure was carried out in a quiet, silent and twilight room in order to limit the stimulations that could alter the results. The evaluation procedure lasted a maximum of 30 minutes from the time of drug administration, during which cats were monitored to fill an evaluation sheet at predefined time points with specific scores (Figures 1-2). During this phase diagnostic and therapeutic procedures such as blood and urine samples, radiographic examination, were carried out in these groups of cats. Drugs were administered IM with 2.5 ml syringe and 22 G needle (T0). At that time a stopwatch was activated to establish the exact time of the evaluations or occurrence of drugs were administered IM with particular effects, recorded in seconds from T0.

The time of onset of lateral recumbency, indicative of sedation, was noted and response to injection was evaluated with scores between 0 (none) to 4 (prolonged reaction).

Physiological parameters included heart rate (HR), respiratory rate (RR), rectal temperature (Temp °C), oxygen saturation of haemoglobin (SpO_2), presence of mydriasis. Parameters were evaluated at predetermined time points and beside physiological data, behavioural parameters reflective of sedation degree were scored.

Date: __________
Patient name: ____________________ M F / I N
Procedure: ____________________

Treatment group random # ______
Opioid: ______________________ *
*Supraspinatus / Quadriceps – ri / le**

Pediatric (0-3 months)
Young (4-12 months)
Adult (>12 months)
Geriatric (>o=10 years)

Time of IM injection_____
Time of onset of lateral recumbency ______

Alfaxalone: 3 mg/kg = _______ (ml) +
a) butorphanol: 0.3 mg/kg = _______ (ml)
b) methadone: 0.3 mg/kg = _______ (ml
c) pethidine: 5 mg/kg = ________ (ml)

Body weight: _____
ASA STATUS: 1 – 2 – 3 – 4 – 5 – E
BCS: __/5

max IM dose 0.5 ml/kg

		pre	3′	5′	8′	12′	15′	20′	25′	30′
Temperament score (pre sedation)	- Very friendly	1								
	- Friendly but reserved/nervous	2								
	- Confident/wriggly	3								
	- Mildly aggressive	4								
	- Very aggressive	5								
Response to injection	- None	0								
	- Mild flinch/tense	1								
	- Flinches away from needle	2								
	- Vocalizes/attempts to escape	3								
	- Prolonged reaction	4								
Overall sedation	- Excellent		1	1	1	1	1	1	1	1
	- Good		2	2	2	2	2	2	2	2
	- Fair		3	3	3	3	3	3	3	3
	- Inadequate		4	4	4	4	4	4	4	4
Recovery quality	- Excellent	1	Comments:							
	- Fair	2								
	- Poor	3								

*= To be crossed ONLY at the end of the procedure for record purpose – keep the observer blinded to the treatment!!

Figure 1. Evaluation sheet with patient data, completed before sedation

PHYSIOLOGIC DATA

	basal	3′	5′	8′	12′	15′	20′	25′	30′										
HR																			
RR																			
Temp C°																			
SpO_2																			
SAP																			
Mydriasis (y/n)																			

Whenever possible!

SEDATION SCORE

		3	5	8	12	15	20	25	30
Position	- Completely alert, able to stand and walk	0	0	0	0	0	0	0	0
	- Sedated, standing or sitting	1	1	1	1	1	1	1	1
	- Lying down in sternal, looking around, able to react quickly, stand up	2	2	2	2	2	2	2	2
	- Lying down in sternal/lateral, reacting slowly, difficult standing	3	3	3	3	3	3	3	3
	- Lying in lateral/sternal, head down, unable to stand	4	4	4	4	4	4	4	4
Resistance to lateral recumbency (if in the cage, roll it)	- Strong	0	0	0	0	0	0	0	0
	- Moderate	1	1	1	1	1	1	1	1
	- Slight	2	2	2	2	2	2	2	2
	- No	3	3	3	3	3	3	3	3
Degree of muscular relaxation	- Poor	0	0	0	0	0	0	0	0
	- Moderate	1	1	1	1	1	1	1	1
	- Good	2	2	2	2	2	2	2	2
	- Excellent	3	3	3	3	3	3	3	3
Response to noise (a kiss-like sound 1m away from the cat)	- Normal response (turns head)	0	0	0	0	0	0	0	0
	- Moderate response (pronounced ear flick)	1	1	1	1	1	1	1	1
	- Weak response (slight ear flick)	2	2	2	2	2	2	2	2
	- No response	3	3	3	3	3	3	3	3

Score= **13** maximum sedation;
if >5→ start procedure;
if <5 after 15′→ 3 μg/kg dexmedetomidine IM (____μg), if inadequate after further 10′→ out of study / add something else (________)
Time of start of the procedure: __________
Catheter placement: not required yes - impossible - placed after dex
Intubation (prior instillation of the larynx with lidocaine): not required yes, with ______ ml Propofol (mg ______)
Other anesthetics required to perform the procedure: ____________________

Figure 2. Evaluation sheet with physiologic data and sedation score

Other information collected from each case included: catheter placement (if not required, if placed without any other drugs or after 3 µg/kg dexmedetomidine IM); orotracheal intubation (if not required or possible only after propofol); other anaesthetics needed to perform the procedure. Independently of the procedure but recording whether surgery was followed or not, recovery was scored from 1 (excellent) to 3 (poor), reporting any possible side effect.
The procedure only started if the sedation score was > 5.
Ordinal variables were evaluated with generalized linear models using a multinomial distribution and cumulative logit as a link function. For repeated variables, time was entered as a covariate, while BCS, age, ASA status and surgery were inserted when appropriate. The results are expressed as an odd ratio (OR) with 95% confidence interval (CI) and P-value. Quantitative variables were analyzed using mixed linear models evaluating the effect of the molecule (3 levels) and, for repeated variables, the impact of time (8 levels) and interaction. The basal value of each variable was entered as a covariate. The sidak correction was used for multiple comparisons. The significance was placed for P<0.05. The analyzes were conducted with software SPSS version 23 (SPSS Inc, Chicago, IL).

RESULTS AND DISCUSSIONS

For this study, we recruited 50 females (20 neutered and 30 intact) and 68 males (22 neutered and 46 intact). Mean body weight was 3.8±1.2 kg. Distribution according to age included more than 50% of adult cats (Table 1). Some relevant results of the recorded parameters are reported in Table 2. Most of the cats were very friendly or friendly but reserved, only 2.5% was very aggressive.
The mean time required before starting the procedure was 420±25 seconds, while the duration of the procedure was 1239±46 seconds. Orotracheal intubation was required in 52% of cats (50% in group M, 48.6% in group B, 43.2% in group P) with concomitant use of 2.7±0.2 mg/kg propofol. Surgery was done in 59.3% of cases; diagnostic and therapeutic procedures were performed in 40.7% (Table 2).

Table 1. Demographics data of the study population

GENDER n (%)	**FI**	30 (25.4%)
	FN	20 (16.9%)
	MI	46 (39.1%)
	MN	22 (18.6%)
BODY WEIGHT kg (mean±SD)		3.8±1.2
BCS n (%)	**1**	1 (0.9%)
	2	15 (12.8%)
	3	72 (61.5%)
	4	27 (23.1%)
	5	2 (1.7%)
AGE n (%)	**Pediatric**	2 (1.7%)
	Young	40 (33.9%)
	Adult	62 (52.5%)
	Geriatric	14 (11.9%)

Table 2. Results of some parameters

TEMPERAMENT SCORE n (%)	**1**	49 (41.5%)
	2	42 (35.6%)
	3	15 (12.7%)
	4	9 (7.6%)
	5	3 (2.5%)
INTUBATION n (%)	**no**	61 (52%)
	yes	56 (48%)
SURGERY n (%)	**no**	48 (40.7%)
	yes	70 (59.3%)

Catheter placement was possible in most cases, but in 3% of cats of group M, B and P was impossible, and in 9% of cats of group B was possible after use of dexmedetomidine IM.
The need to use other anaesthetics to perform the procedure occurred in 31% in group M, 50% in group B and 39% in group P.
Statistically significant differences were found in recovery quality (P < 0.01), with poor scores (score 3) in group B (P < 0.01) and group P (P = 0.05).
However, good recovery (score 2) occurred in most cases with a prevalence of 57.1% in group M and 68.4% in group P. Recovery quality was also analysed in association with BCS, categorized into three levels, and age.
The quality improves with increasing BCS and gets worse in adult and young cats compared to pediatric patients (Tables 3-4).
Patients classified as ASA 1-2 became recumbent more slowly than ASA 3 and ASA 4-5 (P = 0.019) (Table 5*)*.

Table 3. Recovery quality according to age

Parameter	95% Wald Confidence Interval for OR		P-value
	Lower	Upper	
AGE			0.176
Young vs pediatric	1.133	56.248	**0.037**
Adult vs pediatric	1.249	49.217	**0.028**
Geriatric vs pediatric	0.798	53.848	0.080

Table 4. Recovery quality according to BCS categorized into three levels

RECOVERY QUALITY n (%)	BCS			P value
	1-2	3	4-5	
1	1 (6.3%)	23 (31.9%)	10 (34.5%)	0.068
2	12 (75.0%)	43 (59.7%)	17 (58.6%)	
3	3 (18.8%)	6 (8.3%)	2 (6.9%)	

Cats in group B achieved lateral recumbency more slowly than those in group M and group P (Table 5).
Influence of BCS influences the time in which the subjects become recumbent with more significant impact in animals with values placed at the extremities of this scale (Table 5).

Table 5. Influence on the time of lateral recumbency Values with the same letter in each subcolumn are statistically not relevant.

Parameter		TIME OF ONSET OF LATERAL RECUMBENCY (sec)	P-value
OPIOID	**Group M**	222±23	0.359
	Group B	270±37	
	Group P	200±22	
ASA STATUS	**1-2**	252^{a}±21	**0.019**
	3	169^{b}±18	
	4-5	157^{ab}±39	
BCS	**1-2**	240±60	0.180
	3	204±15	
	4-5	275±41	

About physiological parameters, only HR and SpO_2 were influenced by the opioid used ($P < 0.01$). HR was maintained within physiological ranges in cats of group P compare to group M ($P < 0.05$) with mean values of 169±6, 171±6 and 162±6 (in P, M and B group respectively).
HR and RR were influenced by ASA status: HR was lower in cats classified as ASA 1-2 (157±2) compared to ASA 3 (158±3) and ASA 4-5 (166±7); RR was lower in cats classified as ASA 3 (24±1) compared to ASA 1-2 (28±0) and ASA 4-5 (28±2). The rectal temperature didn't change in the three study groups.
Regarding the sedation score, only the degree of muscular relaxation and the overall sedation were influenced by the opioid ($P < 0.05$) and ASA status ($P < 0.001$). The degree of muscular relaxation was poor (score 0) in group B compared to group M, overall sedation was poor (score 3) in group B compared to group M. This latter parameter was excellent (score 0) in cats classified as ASA 3 compared to ASA 1-2.
In agreement with other studies, reported in the literature, we recorded side effects typically described after alfaxalone, such as opisthotonos, hyperextension of the limbs and neck, myoclonus, pedalling, ataxia and vocalizations at recovery (Figure 3).

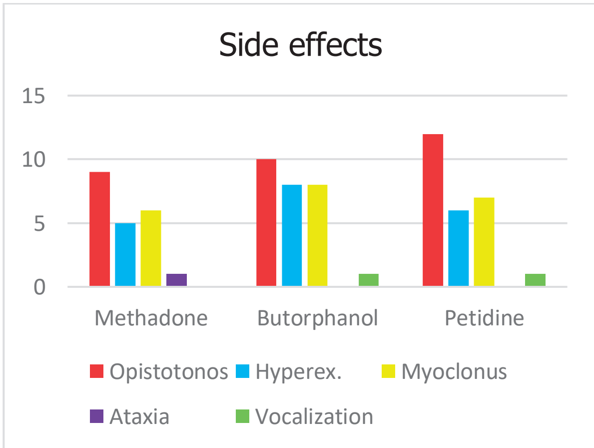

Figure 3. Side effects during the study

CONCLUSIONS

The protocols used in this study was considered safe due to the excellent cardiorespiratory stability and trifling side effects and efficient due to the good sedation effect.
Moreover, most patients were slightly intolerant by the large volume of injection and required adequate physical restraint since alfaxalone is only available at 10 mg/ml formulation.
Lateral recumbency was achieved unexcitedly and in a short time. It was judges as excellent as premedication for patients undergoing general anaesthesia before surgery, but it was evaluated as not entirely suitable for radiographic and CT examinations or blood and

urine samples, that required excessive stimuli. However, in these cases, the administration of a low dose of dexmedetomidine in cats (3 μg/kg IM) was enough to achieve deeper sedation without interfering with physiological parameters evaluated.

Data showed that the best association was alfaxalone-methadone, with greater sedation quality in the shortest time and best recovery, without the use of other anaesthetics. The combination alfaxalone-pethidine showed good results, but more attention is required at the IM injection because accidental IV administration of the opioid could induce an anaphylactic reaction. The association with butorphanol was the least effective of the three in terms of efficacy of sedation and recovery.

Regarding the choice of the opioid used in combination with alfaxalone, it is imperative to consider the patient's age, BCS and ASA status, but also the environment (absence of external stimuli) and the procedure to be performed.

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ANALYSIS OF THE INFLUENCE OF A NUTRACEUTICAL SUPPLEMENT WITH PROBIOTIC EFFECTS ON HEALTH INDEX AND PRODUCTIVE PERFORMANCE IN BROILER CHICKEN

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Abstract

This study was motivated by the fact that the available data on the effects of probiotics and nutraceuticals concerning the health and the productive performance indices in avian species are still controversial. The research consisted in evaluating the influence of BioLactorom nutraceutical product on the main haematological, clinical and biometric indices in Broiler chickens (Ross 308), divided in a control group (n=20) and an experimental group (n=20). According to the manufacturer's instructions, 1 ml of BioLactorom/l water was administered to the chickens in the experimental group in the first 3 days of life, respectively in days 17-19, to reduce the stress generated by manipulation and batching. The chicks were clinically monitored throughout the evaluation, and haematological and microbiological examinations (cultural, on simple agar and McConkey agar, respectively bacterioscopic exam) were performed. All the data obtained emphasized the beneficial effects of BioLactorom, in improving the growth factor, the immunity and the balance of the intestinal flora, and also in reducing the costs of vaccines and other drug products.

Key words: *broiler chickens, nutraceuticals, probiotics, haematological analyses.*

INTRODUCTION

The main purpose of this study, undertaken at the request of ROMVAC Company, was to carry out documentation and investigations to evaluate the safety, tolerance and bioactive potential of the nutritional product BioLactorom (*Lactobacillus plantarum* germs, NCIMB 11974 strain, minimum 1 x 108 CFU/ml, in deproteinized glycerinated whey). With all the remarkable progress made by poultry farming in the last decades, with the expansion of broiler hybrids, the susceptibility of chickens to the major pathogens and precarious environmental conditions has also increased (Huyghebaert et al., 2011), the action of different stress factors becoming a major problem in the intensive poultry farms (Lara and Rostagno, 2013). According to the consulted bibliographies, there is little data available regarding the influence of the nutraceuticals on the blood aspect in broilers raised in intensive system, which fully justifies the investigations carried out in this study. Moreover, there is brief data available on the effect of probiotics and nutraceuticals (a food or part of a food that allegedly provides medicinal or health benefits, including the prevention and treatment of disease) on growth enhancement and production in avian species, as well as on immune system stimulation.

MATERIALS AND METHODS

First, we have to mention that this study was carried out with the Bioethics Commission of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca approval.
Organization of the experiment. The research consisted in evaluating the influence of BioLactorom on the main haematological, clinical and biometric indices in broiler chickens. The biological material (Figure 1) was represented by Broiler (Ross 308) chickens (n=40), one day old, divided in a control group (n=20) and an experimental group (n=20). The chickens were raised on the ground under the

following conditions: kennels with an area of 2 m^2/batch, natural sawdust and daily sanitation; food and water *ad libitum*; 24/7 lighting regime with neon lamps and infrared lamp heating. Initially, crumbled food was administered, containing corn, soy, wheat, vitamin-mineral supplements (calcium carbonate, monocalcium phosphate, oils, L-methionine, sodium chloride, L-lysine, Sacox premix 120, L-threonine, phytase, NSp enzyme) and coccidiostatics (Salinomycin-NA), and after the 18th day with a flour mixture, without the addition of coccidiostatics.

Conduct of investigations. The implementation of the BioLactorom product went through the following sequences: day 0, organization of the study; days 0-3, the first administration of the product (1 ml/l drinking water, for 3 days) and the first weight assessment, following new weighings every week; days 17-19, collection of blood samples for haematological and serological analyses and faecal samples for microbiological examinations, second administration of the product to reduce the stress generated by handling and collecting samples; day 31, the second collection of individual blood samples, respectively individual and mean faecal samples; day 45, completion of the study, including the last collection of blood and faecal samples, as well as repeating clinical and paraclinical examinations.

Figure 1. The appearance of Broiler Ross308 chickens, one day old, when they were introduced to the test

Location and testing period. The investigations were carried out during May-August 2019 and started with the acquisition of the biological material, from a breeding farm of Crăiești-Mureș Avicola Company, following that all the investigations are carried out in the biobase and the laboratories of Physiology, respectively of Microbiology of the Faculty of Veterinary Medicine Cluj-Napoca.

Materials and equipment used. The necessary for this study was composed of the BioLactorom product already presented, the appropriate equipment and consumables. The equipment used included: spectophotometer, centrifuge, drying stove, vortex, colony counting apparatus, microscopes. We also used a set of materials made up of: EDTA and heparin vacutainers, sterile vials for coprology sample collection, gloves, sterile pharyngeal exudate swabs, sanitary alcohol, 5 ml syringes and 22-24G needles, hemocytometers, Natt-Herrick solution, ependorf tubes, well plate, semiautomatic pipettes, ammonia solution, NaCl supersaturated solution, Willis glasses, simple agar plates, McConkey agar plates, gas bulb, loop, degreased blades, DiaQuick Panoptic and May Grunwald-Giemsa dyes; scales, water and feeding pots, bedding, infrared light bulbs.

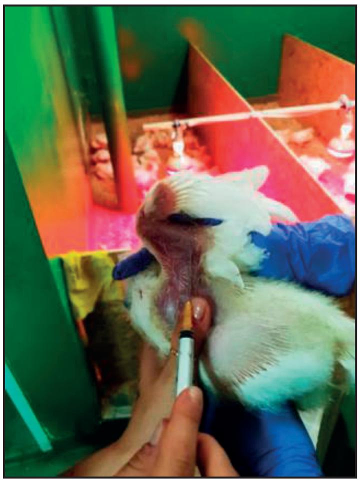

Figure 2. Detail regarding the labor of collecting the blood sample from the basilar vein

Determination of the total number of red blood cells (RBC) and leukocytes (WBC). The count of RBC in the bird shows some differences from the count of RBC in mammals. Prochaska-modified Natt-Herrik dilution fluid is used, which protects all the figured blood elements (erythrocytes, leukocytes, platelets), being considered the standard method (Ognean and Cernea, 2011; Pierson, 2000; Campbell et al., 2007). The working procedure consisted of taking 2 µl of blood from each sample and homogenizing it in an ependorf tube with

400 µl Natt-Herrick solution to obtain a dilution of 1 to 200. After 3 minutes of samples are loaded the hemocytometer and left to settle for 5 minutes. It followed the counting of the WBC in the 4 squares of order I in the corners of the hemocytometer and the count of the red blood cells in 5 squares of order II in the central network (Ognean and Cernea, 2011; Samour, 2006).

Hemoglobin dosage. This determination is difficult due to the presence of the RBC nucleus in the case of blood samples from birds (Hawkey and Samour, 1988; Samour, 2006; Campbell et al., 2007). In order to determine the hemoglobin concentration in birds, the colorimetric method and semi-automatic or automatic methods can be used. We used the semi-automatic spectrophotometric method (Ognean and Cernea, 2011). Thus, we diluted 200 µl of blood with 400 µl of ammonia solution and allowed to stand for 3 minutes, then transferring 20 µl into ELISA-type wells. This determination requires a negative control (ammonia solution) and a positive control (a blood sample whose exact hemoglobin value is known). The reading is done with the help of the spectrophotometer. The result obtained is multiplied by the correction factor 33 for the expression in g/dl as a unit of measurement (Samour, 2006).

Determination of hematocrit. This basic analysis in avian hematology allows a relevant evaluation of the erythrocyte mass. For this purpose we resorted to the evaluation of the macro-hematocrit, in the case of blood samples centrifuged at 2500 rotations/minute and its percentage expression (Ghergariu et al., 2000; Ognean and Cernea, 2006; 2011).

The determination of the erythrocyte constants was based on the use of calculation formulas known in the field (Ghergariu et al., 2000; Samour, 2006; Ognean and Cernea, 2011), these indices being important for birds, especially for the detection of stress of nutritional origin. MCV (mean erythrocyte volume) is an index of cell size and represents the volume occupied by a single red blood cell and has as its unit of measurement femtoliter (fl) (Ghergariu et al., 2000; Ognean and Cernea, 2006; 2011). His knowledge allows the early detection of anemia (Duguy, 1970). MCH (mean eritrocyte hemoglobin) is a color index, which refers to the average amount of RBC hemoglobin. Together with MCHC and other erythrocyte indices, it helps to differentiate between different types of anemia, being expressed in peak grams (pg). MCHC (mean hemoglobin concentration) represents the average hemoglobin concentration in a given volume of erythrocytes, or the ratio of the amount of hemoglobin to the volume of red blood cells and is expressed in g/dl (Ghergariu et al., 2000; Ognean and Cernea, 2006; 2011).

Leukogram determination. Coloring of bird blood smears is based on the use of most Romanovsky-type stains used in mammalian smears (Wright, Gimsa, Wright-Gimsa, Leishman, Wright-Leishman, May-Grunnwald, May-Gundwald-Gimsa, DiaPanoptic etc.). For fastness reasons, in this study we used DiaQuick Panoptic staining, based on the use of 2 dyes (acidophilic and basophilic) and a fixative containing absolute methyl alcohol (Campbell, 1994).

Microbiological analysis of faecal samples. Studies in the field have already demonstrated the positive effects of probiotics on productive performances in animals, mainly stimulating growth and development. It is worth mentioning, however, the beneficial effects of probiotics on increasing resistance to various pathogens, by supporting the proper functioning of the immune system. Starting from the aforementioned, in this study we summarize the quantification of microorganisms content in faecal samples in and establish the dominant taxon. For this purpose, faecal samples were collected, by cloacal sampling, using sterile swabs for pharyngeal exudate, from 5 individuals of each lot. Faecal samples from the litter were also collected for the coproscopic examination of each lot. From the samples taken seedings were carried out on simple agar and McConkey agar, and the resulting cultures, after 12 hours incubation at 30ºC, were used to count the colonies with an automatic microbial colony counting apparatus (Colony Counter CC1).

The populations of intestinal microorganisms were quantified by dilution method, using the microbiological method of determining the number of germs (Zarnea, 1984). For this purpose, 2 mg of faecal sample was added to 1 ml of distilled water, with a dilution of 1:

100,000. The samples thus prepared were vortexed and with the aid of a seeding handle, under the protection of the gas bulb, other series of Petri dishes were seeded on simple agar and McConkey agar, the latter being thermostated at 30ºC for 12 hours, following the final count with the help of the Colony Counter CC1. From the colonies obtained after the dilutions, bacterioscopic examinations were then carried out, on Gram method colored smears, in order to identify and differentiate the microorganisms types, grouping them in Gram-positive and negative, as well as according to the main morphological criteria (shape and grouping of vegetative cells). In parallel, smears were also stained by panoptic methods (May Grunwald-Giemsa).

Monitoring of health status and evaluation of growth enhancement. Chicken lots maintained under optimum conditions according to the "Ross308 strain instruction manual", (http://en.aviagen.com/brands/ross/products/ross-308), were monitored at least 3 times/day, evaluating the level of zoo hygiene and health indices, and as the case may be, the use of general semiotic methods. By inspection, the general health and maintenance status of the chicks was continuously monitored. The evolution of the ventilation and lighting degree, the temperature of the floor and water, the consumption of food and water, the quality of the litter, the appearance of the faeces were also monitored. Thus, any changes were noted and monitored in order to eliminate the various risk factors.

Moreover, according to the protocol, we also monitored the weight gain of the chicks through weekly weighings, both individually and in batches, graphically representing the evolution of the weight of the chicks. According to the protocol, starting on day 18 the coccidiostatic administration was discontinued and coproscopic verification examinations were carried out weekly. We mention that, on days 19-22, we reported the occurrence of isolated cases of feces with blood streaks, which led to an intensification of parasitological examinations. In order to perform the co-parasitological examinations, we used the Willis method, which ensures a good concentration of sporocysts, oocysts, spore cysts, oncospheres, nematode eggs (Cozma et al., 2013).

RESULTS AND DISCUSSIONS

Control group results. The research carried out on this group led to the obtaining of a particularly relevant data set, which statistically processed constituted an important benchmark for interpreting the results from the experimental batches. Following the processing and statistical analysis, we have outlined an overview of the individual variations of the main haematological parameters in broiler chickens. By comparing the values obtained in our research with those of the specialized literature, we were able to outline a relevant image on the dynamics of haematological and leukocyte parameters in broiler chickens.

Evolution of haematological parameters. In Tables 1 and 2, the distribution of data obtained for the statistical analysis of the haematological indices in the control group are presented, which were the basis of the subsequent correlation with the reference values in the specialized literature. The determination of the total number of RBC revealed maximum values of 4.39 T/L at the first harvest and minimum values of 1.75 T/L at the third harvest. The mean values obtained for this parameter ranged from 2.35 ± 0.22 to 2.83 ± 1.04 T/L, being within normal ranges (Wallach and Boever, 1983; Douglas et al., 2010). In the same context, the total number of leukocytes evolved, registering averages (0.29 ± 31.18 and 2.65 ± 28.5 G/L) within physiological ranges (Ghergariu et al., 2000; Douglas et al., 2010). The haematocrit showed an average between 21.89 ± 3.15 and 37.5 ± 4.33%, reconfirming that the blood-cell ratio is slightly lower in meat birds compared to laying hens, interpreted in this context the values obtained can be considered normal. Haemoglobin reached the maximum value of 28.21 g/dl in the case of a chicken at the third harvest, a value that we consider high. In contrast, the minimum values were between 6.5 and 15.12 g/dl, with the average at the first harvest of 8.62 ± 1.37 g/dl, the values being within the reference intervals (Ahmed Al- Nedawi, 2018) and 21.76 ± 6.2 /dl at the third harvest, considered a slightly

increased value according to the studied bibliographies.
Erythrocyte constants were also calculated for each batch separately. Thus, MCV had an average value between 84.77 ± 45.13 and 147.56 ± 39.43 fl, MCH recorded maximum values of 90.79 pg, with a minimum of 27.65 pg values between 37.04 ± 7.54 and 82.3 ± 14.65 pg, evolving within the physiological limits, according to Ahmed M. Al-Nedawi (2018). For the third erythrocyte constant MCHC, the mean values between 39.63 ± 6.38 g/dl and 71.87 ± 52.65 g/dl were within the reference ranges. The distribution of leukocyte subpopulations was characterized by oscillations recorded in their physiological intervals, as evidenced by the data presented in Table 2.

Table 1. Distribution of RBC and leukocyte parameters values in the control group

Parameters	RBC (T/L)	WBC (G/L)	Haematocrit (%)	Haemoglobin (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
			Day 17th				
Average	2.83	2.65	22	12.62	84.77	48.94	71.87
St. Dev.	1.04	2.85	8.36	2.2	45.13	17.92	52.65
			Day 31st				
Average	2.35	2.04	21.89	8.62	94.77	37.04	39.63
St. Dev.	0.22	2.30	3.15	1.37	23.38	7.54	6.38
			Day 45th				
Average	2.64	1.29	37.5	21.76	147.56	82.3	57.44
St. Dev.	0.52	3.11	4.33	6.2	39.43	14.65	12.09

Table 2. Distribution of leucogram values in the control group

			WBC counts		
Cells	Heterophile, %	Eosinophils, %	Basophils, %	Lymphocytes, %	Monocytes, %
			Day 17th		
Average	48.6	2.6	-	39.2	9.6
St. Dev.	8.35	1.94	-	7.91	2.07
			Day 31st		
Average	46.4	4.4	-	37.2	11.8
St. Dev.	10.73	1.14	-	7.01	5.01
			Day 45th		
Average	42.8	3.4	-	40.2	13.6
St. Dev.	12.63	1.67	-	8.4	8.76

Thus, the analysis of the evolution of heterophiles revealed reaching the maximum values of 57-58% and averages of 42.8 ± 12.63 - 48.6 ± 8.35%, within physiological limits. The evolution of eosinophils was also framed by mean values between 2.6 ± 1.94 and 4.4 ± 1.14% and within the physiological intervals (Ghergariu et al., 2000). The basophils were sporadically reported and we did not attach any particular significance to the bird's immune status. The lymphocytes showed maximum values between 47-50% and minimum values between 27-31%, being slightly below the physiological limit. In contrast, the mean values, between 37.2 ± 7.01% and 40.2 ± 8.4%, were within the physiological limits after Hoffmann (1961). Regarding the proportion of monocytes, a maximum value was registered in the case of an individual at the third harvest (29%), this value being considered increased by most bibliographic sources. In contrast, the minimum value was 7%, the averages being in the range 9.6 ± 2.07% and 13.6 ± 8.76%, included in the physiological spectrum (Pârvu et al., 1984; Glystorff, 1983).

Experimental group results. The evolution of the haematological parameters in the chicks from the experimental group, fed with the addition of BioLactorom during the study, are presented in Table 3. In this case, the distribution of the haematological parameters was characterized by large oscillations, within the physiological limits or with insignificant deviations. Thus, the average values of haematocrit and haemoglobin were within the physiological limits (22.00-48.00%, respectively 5.58-15.10 g/dl). In case of the total number of RBC, the mean values (2.27 ± 0.38 - 4.07 ± 5.15) were also recorded within the physiological limits (3.00-5.5 T/L). At the first harvest, in the case of an individual, a maximum value of 13.3 T/L was noted. For the total number of leukocytes the dominant trend was decreasing with a maximum value at the first harvest of 24.0 G/L and minimum values of 8.0 G/L at the last harvest, but without significantly exceeding the normal limits (4-13 G/T). Mean levels of erythrocyte constants showed significant but circumscribed fluctuations between baseline limits for MCV 112.52 ± 17.73 (72.25-173.85 fl), and MCH 46.8 ± 9.59 (19, 6-58.69 μg), compared to references (21.48-34.84 g/dl) the MCHC values being slightly increased in some of the chicks (66.6 g/dl). Data on the distribution of leukocyte subpopulations showed average values of heterophiles proportion of 42.4 ± 4.27 - 50.8 ± 5.21%, ranging between physiological limits (13.00-49.00%).

Table 3. Distribution of RBC and leukocyte parameter values in the experimental group

Parameter	RBC (T/L)	WBC (G/L)	Haematocrit (%)	Haemoglobin (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
				Day 17th			
Average	4.07	18.72	26	13.84	117.63	61.79	54.62
St. Dev.	5.15	13.38	5.47	1.98	58.34	27.93	9.95
				Day 31st			
Average	2.27	19.36	25	10.69	112.52	46.8	42.76
St. Dev	0.38	43.22	0	3.44	17.73	9.59	13.76
				Day 45th			
Average	3.822	10.4	38	19.86	109.48	56.09	52.52
St. Dev	1.35	1.60	4.47	2.73	39.45	14.69	6.31

Table 4. Distribution of leucogram values in the control group

			WBC counts		
Cells	**Heterophile %**	**Eosinophils %**	**Basophils %**	**Lymphocytes %**	**Monocytes %**
			Day 17th		
Average	42.4	4.2	-	43	10.2
St. Dev.	4.27	3.03	-	8.03	2.38
			Day 31st		
Average	50.8	3.6	-	35.4	10.2
St. Dev	5.21	1.51	-	4.72	4.08
			Day 45th		
Average	46	2.6	-	45.4	6
St. Dev	9.51	1.51	-	8.64	2.91

The population of eosinophils evolved likewise and did not exceed the upper limit of 14%, the highest level of 9% being recorded at the first harvest. The dynamics of the lymphocyte population was also characterized by minor variations, from 29% to 55%, within the physiological limits (31.00-72.00%). In contrast, the proportion of monocytes was characterized by wider oscillations (2-17%), still within the reference limits (2.00-16.5%). It is important to mention that the data obtained in the researches on broiler chickens, especially the haematological ones, are characterized by wide variations depending on age, sex, or even circadian. Thus, the mean values of the total number of RBC in the control group (2.35 ± 0.22 - 2.83 ± 1.04 T/L) were slightly lower than in the experimental group, (2.27 ± 0.38 - 4.07 ± 5.15 T/L), being within the normal limits for both lots. Comparatively, the total number of leukocytes in the control group recorded slightly higher levels (1.29 ± 31.18 - 2.65 ± 28.5 G/l) than the experimental group (1.0416-1.93 ± 43.22 G/L), caused by the higher values of some individuals in the control group. Regarding the evolution of heterophiles, proximate average values were also recorded for the control and experimental group (42.8 ± 12.63 - 48.6 ± 8.35%, respectively 42.4 ± 4.27% - 50.8 ± 5.21%), within physiological limits, according to Hoffman (1961), while other authors consider these values to be slightly above the upper limit. Eosinophils showed normal values for both groups (2.6 ± 1.94% - 4.4 ± 1.14%, respectively 2.6 ± 1.51 - 4.2 ± 3.03%). The basophils, were sporadically encountered in the case of both batches, being below the limits recorded in the consulted bibliographies, without having a special significance regarding the health of the birds. The average values of lymphocytes were also within the physiological intervals for both groups (37.2 ± 7.01-40.2 ± 8.4% respectively 35.4 ± 4.72-45.4 ± 8.64 %), according to Hoffmann (1961). Also, the average values of the monocytes were within the physiological limits, both in the case of the control group (9.6 ± 2.07 – 13.6 ± 8.76%), as well as of the experimental group (6 ± 2.91 to 10.2 ± 2.38%). There were also slightly above the physiological limits recorded values in the case of several individuals from the control group, towards the end of the experiment. However, we considered that, as a whole, they were within the physiological limits (Gylstorff, 1983). The haematocrit of the control group had average values between 21.89 ± 3.15% and 37.5 ± 4.33, and for the experimental group between 25% and 26%, without being outside the physiological limits, and only a few individuals in the control group had closer to the lower limit values. Hemoglobin recorded mean values between 8.62 ± 1.37 and 21.76 ± 6.2 g/dl, for the control group, and for the experimental group between 10.69 ± 3.44 g/dl and 19.86 ± 2.73 g/dl. According to Bounous and Stedman, 2000, we consider that these values also fall within the normal intervals, except for a few individuals from the experimental group at which they slightly exceeded the upper limit. Regarding the values of the erythrocyte constants, for the control group, MCV had an average value between 84.77 ± 45.13 and 147.56 ± 39.43 fl, the value of the experimental group was 109.48 ± 39.45 and 117.63 ± 58.34 fl. The MCH recorded mean values between 37.04 ± 7.54 and 82.3 ± 14.65 pg for the control group, and for the experimental group the values were 46.8 ± 9.59 pg and 61.79 ± 27.93 pg. MCHC had an average value of between 39.63 ± 6.38 g/dl and 71.87 ± 52.65 g/dl for the control group and 42.76 ± 13.76 g/dl and 54.62 ± 9.95 g/dl, for the experimental group. We observe that the values of the control group are higher than those of the experimental group, but all the values exceed the upper physiological limit. According to some studies, there is a negative relationship between MCH, MCHC and the total number of RBC, so it is normal for a high amount of hemoglobin to increase the value for MCHC while the total number of RBC is maintained normal.

Microbiological investigations results. All microbiological examinations in the case of the control group, revealed the predominance of the bacteria in the genus *Bacillus* of Gram-negative germs (80%) compared to the Gram-positive ones (40%), as well as the presence of lactose-negative and coliform bacteria. Morphologically, the colonies formed were extremely diverse: large, medium and small, smooth or mucous (Figures 3 and 4). At the second harvest, the colonies were counted using the counting apparatus, obtaining a maximum number of 860 million colonies for chicken M1 and a minimum number of 430 million colonies for M2, the average of the whole lot was 645.08 ± 214.9 (Table 5).

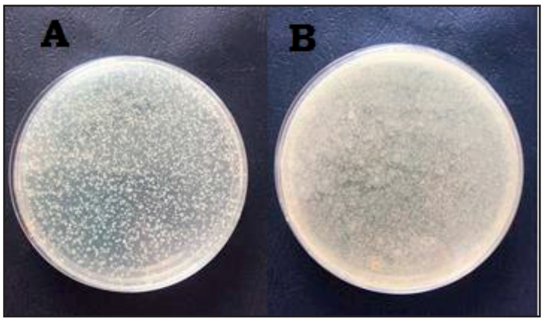

Figure 3. The morphological aspect and density of the developed colonies on simple agar in the experimental group (A) and the control group (B)

Regarding the experimental group supplemented with BioLactorom, only medium and small colonies were identified, and in addition to the control group, the presence of thin filaments belonging to the genus *Lactobacillus* was reported. Also, all the microbiological examinations revealed, for the experimental group, the predominance of Gram-positive bacilli (100%), while the Gram-negative bacilli and cocobacilli were in a much smaller proportion (60%). In the case of the test group, there were no large, mucous colonies, which often merge together, the pathogenic germs being most often responsible for their formation. Regarding the evolution of the number of colonies, the maximum value of 688 x 10^6 was reached in a chicken in the experimental group, and the minimum value of 113.4 x 10^6 in the case of a chicken in the same group (Figure 3A), with an average of 315.28 x 10^6 ± 228.55. Comparative analysis of the average values recorded in the control group (645 x 10^6 ± 214.9) and in the experimental group (280.08 x 10^6 ± 228.5 (Table 6), with significant differences between the two groups, due to the supplementation of the diet with the investigated nutritional product.

For the control group, we can conclude that, the results of the microbiological examination suggest possible imbalances of the endosymbiotic microflora, the balance being inclined towards the predominance of the Gram-negative germs (80%), while the Gram-positive bacteria had a minor weight (40%).

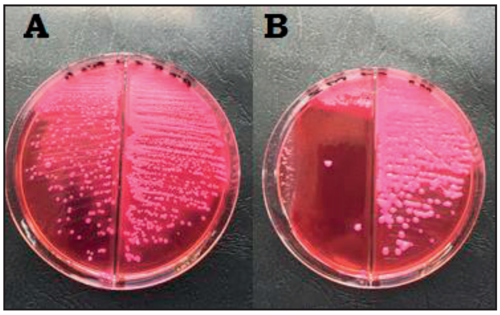

Figure 4. The morphological aspect and density of the developed colonies on McConkey agar in experimental group (A) and control group (B)

The increased number of colonies in the control group suggests an overpopulation of the intestinal tract, an aspect that adversely affects broiler chickens, because the lack of balance of intestinal endosymbionts can lead to the destruction of the epithelial barrier, with negative effects on growth and health in general. We consider that, for the experimental group, the microbiological examination results revealed some significant beneficial effects compared to those obtained in the control group. In this context, we mention that the endosymbionic microflora from the intestinal level is particularly important for the functioning of the defense mechanisms in birds, both the innate and the adaptive ones.

As it is well known, the gut is populated with both organism-beneficial bacteria, such as Gram-positive bacteria, lactobacilli or bifidobacteria, as well as potentially pathogenic bacteria, such as *Clostridium* spp., *Escherichia coli, Salmonella* etc.

Table 5. Distribution of the total number of colonies in the control group

	Parameters	Colony count
M1	Max.	860
M2	Min.	430
M3	Average	645.08
M4	St. Dev.	214.9
M5	Median	645

It is vital to maintain a constant balance between these two categories. Thus, it is considered that 85% of the intestinal bacterial population should be Gram-positive bacteria, so that digestion works well.

Table 6. Distribution of the total number of colonies in the experimental group

	Parameters	Colony count
B1	Max.	688
B2	Min.	113.4
B3	Average	315.28
B4	St. Dev.	228.55
B5	Median	280.8

Based on the obtained results we consider this was fulfilled by the addition of the nutraceutical product BioLactorom, formulated by the Company ROMVAC, in the tested diet. Based on the good growth level achieved we consider the BioLactorom product exerts beneficial effects on weight dynamics (Figure 5) in broiler chickens. In this regard, the results of the microbiological tests obtained for the experimental group were also shown, indicating the importance of a balanced intestinal flora in the processes of digestion, absorption and defense in birds, as well as for reducing the effects generated by stress.

The synthesis of the results obtained in the clinical and coproscopic examinations revealed a good general condition and maintenance of the chicks throughout the experiment, expressed by the increased level of appetite, the temperament, the pleasant aspect of the plumage. We also recall that at the coproscopic examinations, no major pathogens were reported, except for days 20-24 of the experiment, when due to the change of the feeding some chicks presented soft feces with blood streaks.

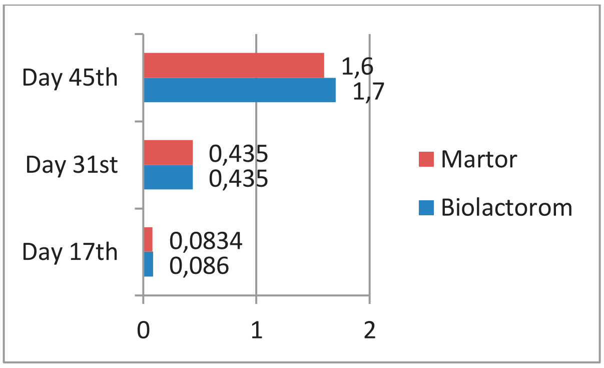

Figure 5. Graphical representation of weight dynamics in control group and fed with BioLactorom chickens

Given that the sub-therapeutic use of antibiotics in feed has been either banned or reduced in most countries (Sugiharto, 2014) due to the fact that they caused the emergence and/or extension of the known phenomenon of "anti-resistance", thus representing a health hazard. For consumers, the use of nutraceuticals in the poultry farming industry is not only increasingly appreciated, but has been used extensively. The exclusion of antibiotics from feed has caused many problems in the industry, as the growth performance decreased and during the breeding period the birds developed subclinical diseases with an increasing mortality rate (Huyghebaert et al., 2011). Moreover, intestinal health has recently been the subject of intense studies aimed at poultry production (Rinttila and Apajalahti, 2013). In addition to improving environmental conditions, nutritional strategies have been developed to partially alleviate the negative impact of stress on birds (Lara and Rostagno, 2013), including diet with high energy density, addition of salts, antioxidant and mineral vitamins in stressed bird diets (Sahin et al., 2009; Das et al., 2011). Recently, dietary supplementation with probiotics, prebiotics and symbiotics has been implemented in poultry to counteract the negative effects of heat stress (Lara and Rostagno, 2013).

We believe that Romvac BioLactorom, a probiotic for the stabilization of intestinal flora, has beneficial effects on growth and stimulating effects on defense mechanisms in birds, even under stressful conditions. However, the evolution of daily growth growth did not reveal the existence of very large differences between the two batches, at the end of the 45 days the average weight of the batch supplemented with the BioLactorom product was slightly higher (1.7 kg) than the control batch (1.6 kg). We can therefore consider that this effect of biometric stimulation is due to the supplementation of the diet with the probiotic investigated, whose action of balancing the intestinal flora has been proved by the microbiological tests performed.

CONCLUSIONS

An important contribution of the nutraceutical BioLactorom has significantly contributed to balancing the intestinal flora and achieving a good level of growth in Broiler chickens and also has been shown to stimulate and support immunity in Broiler chickens (reducing the costs of administering vaccines and medicinal products, including coccdiostatics), expressed by reducing mortality and severe episodes of disease;

Following the administration of the product in the drinking water at a significant number of broiler chickens, the necessity of reformulating the BioLactorom product was outlined, for the administration in feed, which is more suitable for the industrial growth of the birds.

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ADAPTATION OF COTTON (*Gossypium hirsutum* L.) TO LIMITED WATER CONDITIONS: REVERSIBLE CHANGE IN CANOPY TEMPERATURE

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Abstract

Improving cotton yield under limited water supply needs to deeper understanding of the plant's response and adapting strategies to improve their tolerance. Effects of limited water conditions on ten cotton genotypes (Gossypium hirsutum L.) were examined in a field experiment to evaluate their tolerance level and explore time depending changes in canopy temperature and leaf greenness as indirect determinations of leaf water-status and chlorophyll density. Plant height shortened (15%), dry matter accumulation inhibited (36%), ball number (35%) and eventually lint yield (35%) decreased of all cotton genotypes since irrigation amount decreased 32% under limited watering conditions (LWC). Significant genotypic variation in tolerance level and yielding capacity under LWC were found among genotypes. Leaf tissues accumulated higher proline (stress-related amino acid) to adapt lower water potential conditions while canopy temperature depression (CTD) reversibly decreased and SPAD values were increased. A strong correlation between relative changes in CTD and SPAD values and a significant variation in ability of the cotton genotypes to recover CTD under limited water conditions were found. Our results also suggested that the higher ability to recover CTD of cotton leaves was associated with lower total dry weight reduction and water stress susceptibility under limited water conditions.

Key words: *Cotton, water, canopy temperature depression, SPAD, drought, proline.*

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is one of the most important cash crops in Turkey since it provides fibre to textile, edible oil to food and animal feed to livestock industries. It is mostly grown South and South-West coastal and South-East part of Turkey under irrigated conditions. Supplementary irrigation is needed because the natural precipitation is not adequate during growing period of cotton in these locations (Turan and Göksoy, 1995). However, increase in frequency of drought events and restricted water resources are considered as main limiting factor for cotton production in Turkey (Tatar, 2016). Therefore, improving water management and increasing water use efficiency under limited watering conditions will inevitably be a major challenge for sustainable cotton production. However, most of the efforts to improve efficient use of water in cotton production system reduce available soil-moisture content which inhibits plant growth (Chastain et al., 2014; Turner et al., 1986), lint yield and productivity (Wells and Stewart, 2010; Pettigrew, 2004; Krieg and Sung, 1986). On the other hand, cotton genotypes differ in their adaptation ability to limited watering conditions (Sezener et al., 2015; Rahman et al., 2008; Penna et al., 1998). The adaptation ability is both considered as a lower reduction in photosynthetic rate thus total dry matter accumulation or lint yield and productivity under restricted irrigation (Megha and Mummigatti, 2017; Singh et al., 2015).
Stomatal limitation hence reduction in leaf internal CO_2 concentration and net photosynthesis are the primary responses of plants under water stress conditions (Chastain et al., 2014; Loka et al., 2011; Cornic and Fresneau, 2002). Lower transpiration mediated by stomatal closer to conserve limited water content in plant tissues dysfunctions so-called cooling system of the leaves (Wiegand and Namken, 1966) and increases leaf temperature (Wanjura et al., 2004; Wiegand and Namken, 1966). Canopy temperature depression (CTD) which is expressed as differences between ambient air and canopy temperature (Ray and Ahmed, 2015; Smith et al., 1986) has been widely implemented to estimate plant water status (Blum et al., 1982; Idso 1982; Jackson et

al., 1981; Ehrler, 1972). Many studies previously suggested that there is a strong linear relationship between CTD and soil moisture content, transpiration rate and stomatal conductance (Nagler et al., 2003; Inoue and Moran, 1997; Inoue et al., 1994; Moran et al., 1994; Inoue et al., 1990). Karimizadeh and Mohammadi (2011) also reported that maintaining ability of CTD in wheat genotypes under limited watering conditions was significantly associated with tolerance level of the plants and they suggested CTD measurement as a useful indicator for screening stress tolerance.

Considering genotypic variation on stomatal regulation and evaporative cooling capacity of different species under water limited environments, still limited information exists about time depending changes in leaf level regulation of CTD in cotton plants.

Metabolic malfunctioning, disorder of carbohydrate metabolism (Loka and Oosterhuis, 2012) as well as damages in photosynthetic tissues (Shahenshah and Isoda et al., 2010; Bilger and Björkman, 1990; Björkman and Demming-Adams, 1994; Inamullah and Isoda, 2005) are the secondary water stress induced response of plants. Cotton plants as most of the other plant species accumulate proline during secondary phase of the stress (Verbruggen and Herman, 2008) due to its crucial role in osmoregulation, structural protection (Yi et al., 2016) and ROS scavenging (Smirnoff and Cumbes, 1989). Changes in green color of leaves are the most apparent and visual symptom of differentiation in chlorophyll content and density under stress conditions (Conaty et al., 2008; Thongbai et al., 2001; Boquet et al., 1999). Chlorophyll pigments mostly degrade and generally leaves become light-green under limited water conditions (Tatar et al., 2016; Hejnak et al., 2015; Sarani et al., 2014) though a number of the studies report increasing chlorophyll content (Martinez and Guiamet, 2004; Ahmad et al., 2013) which imply reducing leaf area and increasing pigment density. Most of the water stress related studies determine chlorophyll concentration of stress-induced leaves at the end of treatments (Pandey et al., 2003; Patil et al., 2011) and misses the changes during adaptation period. Therefore, non-destructive determination of chlorophyll concentration such as SPAD measurement gives more detail about leaf level adaptation to water limited conditions.

The aim of the study was to (i) determine tolerance level and yield potential of ten cotton genotypes under limited water conditions, (ii) asses leaf level adaptation of cotton under restricted irrigation considering CTD and chlorophyll concentration (iii) and evaluate correlations between the adapting ability of the leaves and the tolerance levels of the genotypes.

MATERIALS AND METHODS

This study was conducted in 2014/15 at the experimental site of Ege University, Faculty of Agriculture, Department of Field Crops, Izmir, Turkey (38°27'6", 27°13'32"E). The soil type of experimental field was clay loam, mild alkaline and moderate calcic. Air temperature (°C) and relative humidity (%) were recorded by a gauge (Tinytag Plus 2®) every 10 minutes and rain amounts (mm) were measured by pluviometer during the growing period of cotton (Figure 1).

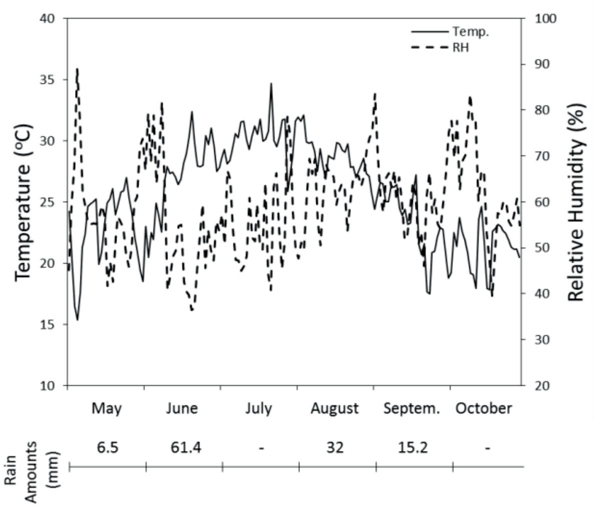

Figure 1. Average temperature (°C), relative humidity (%) and rain amounts (mm) recorded during growing period of cotton in the experimental site

The experiment was comprised of two different irrigations and ten cotton genotypes with three replications in the Randomized Block Design. The plot dimensions were 2.8 x 3 m and the experiment was consisted of 60 plots. Drip irrigation system was settled to the all plots and irrigation amounts were recorded by water flow meter. Row space and sowing distance in lines were 0.7 m and 0.20-0.25 m, respectively. Initially, 100 kg/ha of basal nitrogen as composite fertilizer (15-15-15) and 100 kg/ha as ammonium nitrate (33%) at the beginning of flowering stage were applied. The cotton plants were maintained for dispelling the negative effect of weeds and insects. Commercial insecticide containing malathion were sprayed to the plants to avoid *Aphis* spp. and *Empoasca* spp.

Totally 10 genotypes of *Gossypium hirsutum* L. (ST 468, ARCOT 008, ST 373, ST 498, GAIA, MAY P 06, ARCOT 009, FLASH, GLORIA and PG 2018) which is well adapted to coastal part of Turkey were used in the experiment. Two watering treatments were applied after homogenous plant stands were obtained (4-5 leaves stage). Totally 454 mm of water were received by irrigation in well watering (WW) plots whereas 310 mm in limited watering (LW) treatment.

Youngest fully developed leaves were collected 30 days after treatments for proline analysis. According to Bates et al. (1973), 50 mg of oven dried leaf samples were extracted with 10 ml sulfosalicylic acid solution in mortar and then filtered. Then, 2 ml ninhydrin solution and 2 ml glacial acetic acid were added to extracted sample and boiled at 100°C in water-bath. Then the reaction of the mixture was stopped by ice-bath. When the mixture was reached to room temperature, 3 ml toluene was added to the mixture. Upper layer of the solution was used to determine absorbance values by using spectrophotometer (Carry 50®).

Canopy temperature (CT) was monitored using with infrared thermometer after onset of the treatments. Measurements were held 7 times and 5 CT data were obtained from each plot in every measurement. Then canopy temperature depression (CTD) was calculated according to Ayeneh et al. (2002) by using following equation:

$$CTD = T_A - T_C$$

where:

CTD - canopy temperature depression; T_A - instant air temperature (°C); T_C canopy temperature (°C).

SPAD value were determined using with (Konica Minolta – SPAD-502 Plus) parallel to CTD measurements (7 times after onset of the treatments) The measurements were held in youngest fully developed leaves of randomly selected 5 plants in each plots.

After removing border rows of all plots, plant height, total dry weight and boll number of randomly sampled 10 plants from each plot were determined before harvest. Then all plots were harvested by hand-picking two times during October. The harvested products were separated into lint and seed by rollergin type machine. Cotton lint and seeds were weighed separately and lint percentages were calculated.

Data were subjected to analysis of variance for each parameter. All data were analyzed by using standard ANOVA techniques of Statistica software. The mean values of each parameter were compared according to LSD test described by Steel and Torrie (1980).

RESULTS AND DISCUSSIONS

Plant height, total dry weight, boll number, lint yield of selected 10 cotton genotypes significantly decreased and proline content increased under limited water conditions whereas lint percentage did not remarkably change (Table 1). The highest decrease in total dry weight were found in FLASH (50.0%) while lowest decreases in ARCOT 008 (17.2%) and PG 2018 (21.3%). Reduction in lint yield was also lower in PG 2018 (15.6%) and GAIA (15.4%) under limited watering relative to well-watering conditions. Similar to total dry weight reduction, FLASH had the most drastic decrease in lint yield (48.9%). Limiting water led 1.54 fold increase in proline accumulation of the leaves in average (Table 1). However, proline content increased in all cotton genotypes though no significant genotypic variation was found.

Average of SPAD values for all growing period markedly increased (14.2%) under limited water conditions (Figure 2). Relative increase in SPAD value was more remarkable in MAY P 06 (18.6%) while ARCOT 008 had lowest increase (11.1%).

Canopy temperatures in average were 5.9°C lower than ambient air (canopy temperature depression - CTD) in well-watered (WW) conditions whereas cooling capacity of the leaves under limited water (LW) conditions was 4.3°C (Figure 2). Higher relative increase in CTD under LW conditions was recorded in ST373 and lower in ACROT008.

The quadratic functions of relative change in CTD and SPAD values for each cotton genotypes during growth period were figured and demonstrated as discriminant of polynomial (Δ) which indicates recovery of plants (Figure 3). The highest Δ (8.62 [r = 0.98]) was found in PG 2018 while MAY P 06 had the lowest Δ value (2.74 [r = 0.87]) in relative change of CTD functions. On the other hand, considering relative change in SPAD values under LW conditions MAY P 06 had higher Δ (1.24 [r = 0.92]) whereas GAIA had lower Δ (0.40 [r = 0.81]).

Table 1. Plant height (cm), total dry weight (kg/m^2), boll number (number/plant), lint yield (kg/ha), lint percentage (%) and proline content (mg/g) of 10 cotton genotypes grown under well-watered (WW) and limited watering (LW) conditions

Cultivars	Plant Height (cm)			Total Dry Weight (kg/m^2)			Boll Number (number/plant)			Lint Yield (kg/ha)			Lint Percentage (%)			Proline (mg/g)		
	WW	LW	AVG	WW	LW	AVG	WW	LW	AVG	WW	LW	AVG	WW	LW	AVG	WW	LW	AVG
ST 468	78.5	66.5	**72.5**	0.97	0.55	**0.76**	22.3	15.2	**18.7**	1463	885	**1174**	43.1	44.2	**43.7**	1.45	2.33	**1.89**
ARCOT 008	77.2	73.2	**75.2**	0.87	0.72	**0.79**	22.0	14.4	**18.2**	1164	840	**1002**	40.1	40.8	**40.4**	1.15	1.77	**1.46**
ST 373	90.2	73.2	**81.7**	1.06	0.66	**0.86**	19.3	13.8	**16.6**	1606	1048	**1327**	40.7	41.7	**41.2**	1.02	2.34	**1.68**
ST 498	85.9	72.6	**79.3**	1.11	0.64	**0.87**	16.3	13.6	**15.0**	1659	955	**1307**	42.5	44.0	**43.2**	1.85	2.11	**1.98**
GAIA	82.3	68.9	**75.6**	0.95	0.69	**0.82**	22.1	12.2	**17.2**	1417	1202	**1310**	41.9	43.3	**42.6**	1.41	2.42	**1.91**
MAY P 06	86.6	68.9	**77.7**	1.05	0.67	**0.86**	26.1	15.0	**20.6**	1684	1110	**1397**	42.9	43.7	**43.3**	1.14	2.73	**1.94**
ARCOT 009	80.1	63.7	**73.5**	0.89	0.54	**0.75**	17.8	12.5	**15.7**	1247	680	**964**	44.0	41.2	**42.9**	1.69	1.92	**1.78**
FLASH	84.2	68.7	**76.5**	1.12	0.56	**0.84**	20.1	12.4	**16.3**	1643	837	**1240**	41.3	43.0	**42.2**	1.30	1.96	**1.63**
GLORIA	86.7	75.9	**81.3**	1.12	0.71	**0.92**	22.8	13.1	**18.0**	1629	952	**1290**	42.9	42.9	**42.9**	1.32	2.02	**1.67**
PG 2018	86.1	82.1	**84.1**	0.94	0.74	**0.84**	20.3	11.6	**15.9**	1217	1032	**1124**	43.4	43.7	**43.6**	2.02	2.03	**2.02**
AVG	**83.8**	**71.3**	**77.6**	**1.01**	**0.65**	**0.83**	**20.9**	**13.5**	**17.2**	**1473**	**954**	**1213**	**42.3**	**42.8**	**42.6**	**1.41**	**2.17**	**1.79**

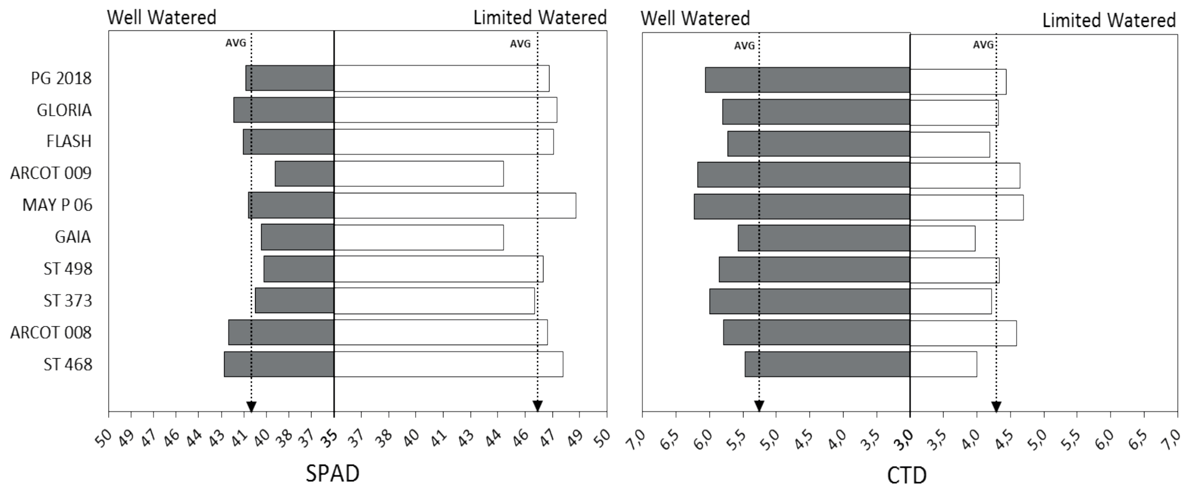

Figure 2. Average values of canopy temperature depression (CTD) and SPAD (totally 7 measurements during adaptation) of ten cotton genotypes under well-watered and limited-watered conditions. Black pointed lines demonstrate average (AVG) values of SPAD and CTD

Correlation between the relative increase in CTD and SPAD values of ten cotton genotypes was demonstrated in Figure 4. The figure indicated that higher relative increase in CTD value was significantly associated with relative increase in SPAD value (r = 0.67).

Relations between recovering ability (Δ) of CTD with relative reduction in Total Dry Weight (TDW) under LW conditions and Stress Susceptibility Index (SSI) are introduced in Figure 5.

Correlation analysis revealed that higher ability of the cotton leaves to recover CTD significantly (r = 0.78) associated with lower total dry weight reduction under limited water conditions. Moreover, stress susceptibility index of the cotton genotypes decreased due to increasing ability of plants to CTD recovery.

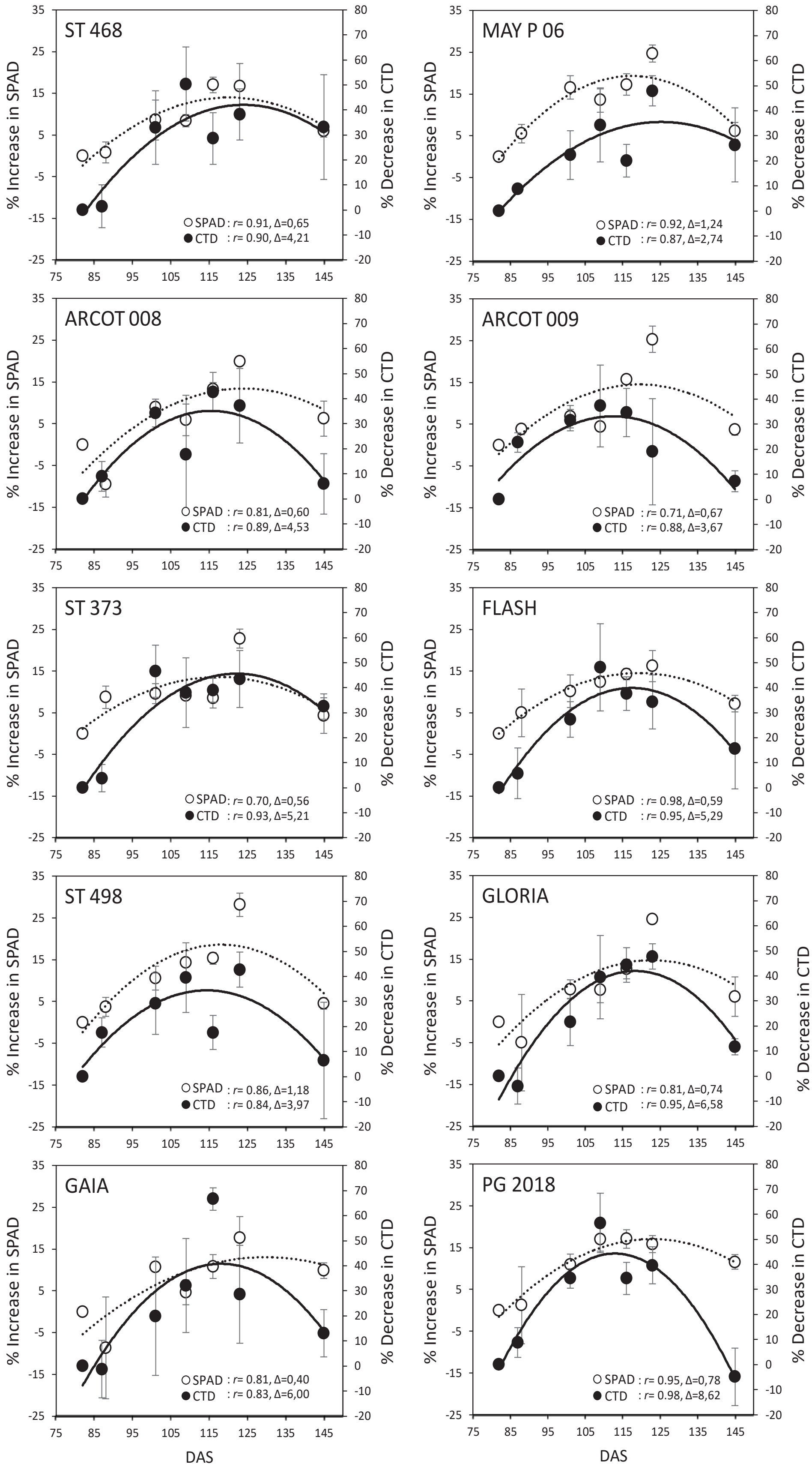

Figure 3. Changes in SPAD and canopy temperature depression (CTD) values under limited-watered condition relative to well-watered conditions of ten cotton genotypes during adaptation period. Delta (Δ) indicates discriminant of quadratic polynomial functions of SPAD and CTD values referring recovering ability of the cotton genotypes (DAS: day after sowing)

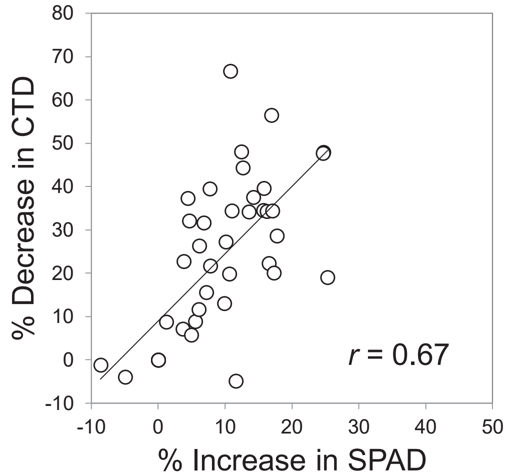

Figure 4. Correlation between relative changes in SPAD and canopy temperature depression (CTD) values under limited-watered condition relative to well-watered conditions of ten cotton genotypes during adaptation to limited watering conditions

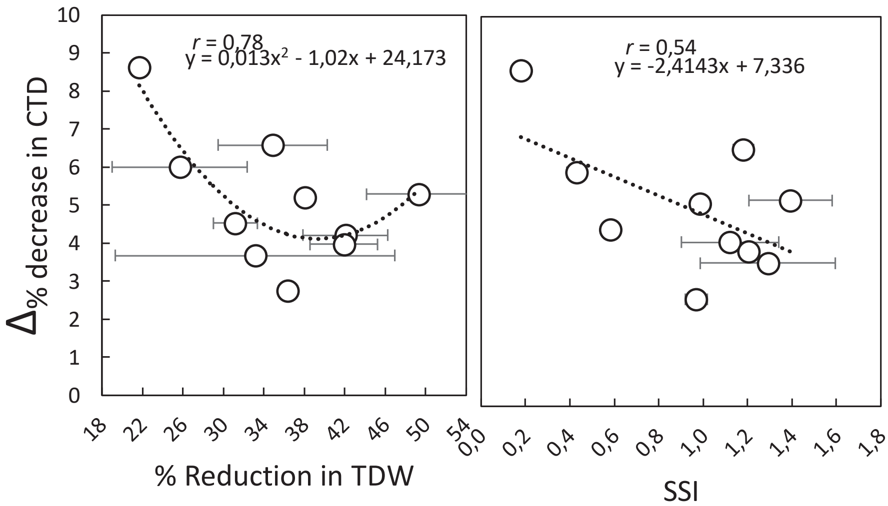

Figure 5. Correlation between relative changes in SPAD and canopy temperature depression (CTD) values under limited-watered condition relative to well-watered conditions of ten cotton genotypes during adaptation to limited watering conditions

Cotton is mostly grown under irrigated conditions in Turkey since the rainfall amount is not sufficient during growing period. According to the future predictions for the cotton growing areas, water resources will be drastically restricted due to climate change within the present century (Tatar, 2016). Therefore, increasing water use efficiency and adaptation to limited watering conditions of cotton is expected to be primary issue for sustainable cotton production. However, limited water conditions often inhibit cotton growth and productivity (Chastain et al., 2014; Pettigrew, 2004; Krieg and Sung, 1986). Our results also indicated that plant height shortened, dry matter accumulation inhibited, ball number and eventually lint yield decreased since irrigation amount decreased 32%. However, genotypic variation was found in productivity of selected cotton genotypes responses to limited water conditions. GAIA could be defined as high-yielding cotton genotype (1.202 kg/ha) under limited-watering conditions comparison to other selected genotypes. Beside the yield performance, PG 2018 was the most tolerant and FLASH was the most sensitive cotton genotypes considering the relative changes in dry matter production and lint yield under limited watering conditions. Ullah et al. (2017) stated that the emphasis should not be only on stress tolerant cotton varieties but also on stability of yield. In order to distinguish this fundamental fact, GAIA might be suggested for limited watering production systems while PG 2018 and FLASH could be referred as contrasting genotypes (tolerant and sensitive respectively) in terms of their responses to limited watering conditions.

SPAD measurement is widely used to estimate chlorophyll content of the leaves (Brito et al., 2011; Istipliler et al., 2016; Tatar et al., 2016). Although SPAD units commonly decreased at the end of the water stress treatments in many studies (Fanizza et al., 1991; Saravia et al., 2016), initial increases in SPAD have been also reported in maize (Martinez and Guiamet, 2004), artichoke (Paungbut et al., 2017) and wheat (Tatar et al., 2016). Increase in SPAD value as an initial response of cotton plants to water limited conditions then lasting decrease could be perceived as leaf level regulation to reduce transpiring area in the present study. We assumed that the reduction in leaf area led an increase in chlorophyll density thus SPAD value during earlier phase of limited watering conditions. Arunyanark et al. (2008) also reported an increase in photosynthetic pigment density in peanut plants under drought conditions. We suggested that the ability of the cotton genotypes reducing leaf area and hence the transpiring surface to conserve water under limited conditions depend largely on the elasticity of the leaf tissues.

The linear relationship between canopy temperature depression (CTD) with soil moisture content, transpiration rate and stomatal conductance have been previously reported in several studies (Nagler et al., 2003; Inoue and Moran, 1997; Inoue et al., 1994; Moran et al., 1994; Inoue et al., 1990). CTD has been also approved as an indicator of plant water status (Karimizadeh and Mohammadi, 2011; Blum et al., 1982; Idso 1982; Jackson et al., 1981; Ehrler, 1972) and used to determine cooling ability of leaves as screening drought responses of plants (Karimizadeh and Mohammadi, 2011; Pinter et al., 1990; Hatfield et al., 1987). The relative decrease then the increase in CTD values of the cotton genotypes under limited water conditions indicated that a reversible adaptation plays a role to maintain transpiration and cooling leaves in the present study. The strong correlation between relative changes in CTD and SPAD values of the cotton genotypes under limited water conditions revealed that leaves became smaller and warmer during initial phase of water limitation then they adapted until a certain level. On the other hand, we demonstrated that there was a significant variation in ability of the cotton genotypes to recover CTD. We may also suggest that the higher ability to recover CTD of cotton leaves is associated with lower total dry weight reduction and water stress susceptibility under limited water conditions.

CONCLUSIONS

Genotypic variation was found in productivity of selected cotton varieties responses to limited water conditions. GAIA could be defined as high-yielding cotton genotype under limited-watering conditions comparison to other selected genotypes. Beside the yield performance, PG2018 was the most tolerant and FLASH was the most sensitive cotton genotypes considering the relative changes in dry matter production and lint yield under limited watering conditions. A strong correlation between relative changes in CTD and SPAD values and a significant variation in ability of the cotton genotypes to recover CTD under limited water conditions were found. Our results also suggest that the higher ability to recover CTD of cotton leaves was associated with lower total dry weight reduction and water stress susceptibility under limited water conditions.

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IN VIVO AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *Cnicus benedictus*

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Abstract

The aim of this study was to comparatively evaluate the in vivo and in vitro Cnicus benedictus L. antioxidant activity based on secondary metabolites (total polyphenolic content and flavonoids). The species is used as an antidepressant, anti-inflammatory, antiseptic, cardiotonic, antimicrobial and anti-proliferative. The samples used for biochemical analysis were represented by two type of in vitro regenerated callus and different parts of in vivo mother plant. The callus cultures were initiated from the leaf explants from potted plant, cultured on Murashige and Skoog (MS) medium supplemented with 2.4-Dichlorophenoxyacetic acid (2.4D) alone or in combination with 6-Benzylaminopurine (BAP). In our conditions, the antioxidant activity was correlated with total phenolic content. The level of flavonoids was higher in callus than in the mother plant. Through HPLC, the rutin presence was validated in callus, and a higher number of constituents were observed.

***Key words**: callus, Cnicus, flavonoids, HPLC.*

INTRODUCTION

One of the most significant parameters which regulate the therapeutic effects of a plant is the antioxidant activity based on bioactive compounds represented by phenolics and flavonoids (Chandur et al., 2011).
The spontaneous medicinal plant species *Cnicus benedictus* L. (blessed thistle, Asteraceae family) has a great potential as an alternative oil crop, being liable to be cultivated on medium-quality soil with low agricultural costs or efforts (Ghiasy-Oskoee et al., 2018). In the past decade, the plant gained more interest, starting to be cultivated as a food source and additive in Turkey (Can et al., 2017) and Romania (Ministry of Agriculture and Rural Development - M.A.D.R., 2011).
The chemical constituents of this plant are represented by sesquiterpene lactone glycosides, cnicin, triterpenoids, lignans, flavonoids, tannins, essential and volatile oils, many nutritional components, minerals and trace elements (Al-Snafi, 2016). The oil of *C. benedictus* is attractive for cosmetics and human nutrition (Horn et al., 2014), being characterised by a high concentration of α-tocopherol (600-750 mg/kg), higher than in sunflower oil (432-730 mg/kg) (Bele et al., 2013; Grilo et al., 2014).
Different aspects concerning scientific literature, expert opinion, folkloric precedent, history, pharmacology, kinetics/dynamics, interactions, adverse effects, toxicology and dosing of this species were presented by Ulbricht et al. (2008). Recent studies about the pharmacological effects of *C. benedictus* are available (Păun et al., 2015; Chabane et al., 2013).
The aim of this study was to evaluate the *in vivo* and *in vitro* antioxidant activity of *Cnicus benedictus*, a medicinal plant species.

MATERIALS AND METHODS

Plant material was represented by *C. benedictus* seedlings obtined from seeds germinated at room temperature. In order to induce *in vitro* callus, leaves fragments were sterilized with $HgCl_2$ 0.1% and cultivated on two MS (Murashige et Skoog, 1962) media variants supplemented with 3% sucrose (w/v), 0.8% agar. In the first variant, MS was added with 1 mg/l 2.4D, and the second variant was supplemented with 1 mg/l 2.4D + 0.2 mg/l BAP. The culture dishes were placed for 30 days in a Weiss Gallenkamp Fitotron, at 25 ± 2°C, with a photoperiod of 16/8 and a 2*36W fluorescent lamp with maximum intensity ~ 90 $\mu mol\ m^{-2}\ s^{-1}$.

Biochemical analyses. The samples used for biochemical analyses were represented by parts of plant (achenes, roots, leaves, stem) and 4 weeks old callus induced *in vitro* from the same plant.

Extraction of *C. benedictus* antioxidant compounds. The samples were dried on filter paper, grinded with quartz sand and extracted in a ratio of 1: 1 (v/v) with 100% methanol for 24 h. The homogenates were centrifuged at 10,000 g, for 20 minutes and the supernatants were used for subsequent analysis.

Determination of antioxidant capacity by DPPH (2.2 diphenyl-1-picrylhydrazyl) method. According to the method proposed by Marxen et al. (2007) 100 µl of diluted (1:2) extract were mixed with 2.25 ml of methanol and 150 µl of 1.27 mM DPPH. In control variant the extract was replaced with the extraction solvent. After 30 minutes of incubation at room temperature, the absorbance at 515 nm was read. Antioxidant capacity was represented by the differences between samples and control against a standard curve that used Trolox (synthetic antioxidant α-tocopherol analogue) as standard antioxidant. Antioxidant capacity was expressed as Trolox equivalents/ fresh weight (mM Trolox/g sample).

Determination of the Total Phenolic Content (TPC). TPC was determined according to the method described by Mihailović et al. (2013) with some changes. The reaction mixture consisted in 0.5 ml of suitably diluted extract (1:2 for calus samples and 1:5 for *in vivo* samples), 2.5 ml of Folin-Ciocalteu reagent 11-fold diluted and 2 ml of 7.5% Na_2CO_3. This was kept for 30 minutes at room temperature, and then absorbance was measured at 765 nm. Three repetitions of the same variant were made and the average represents total phenol content expressed as gallic acid equivalents/ fresh weight (EAG µg/g sample).

Determination of the flavonoids content. Flavonoid content estimation of the methanol extracts was performed using a protocol described by Zhishen et al. (1999), with minor adjustments. Thus, suitably diluted 0.5 ml methanol extract of each sample was mixed with 2 ml of distilled water and 5% $NaNO_2$ solution and the mixture was equilibrated for 5 min. Then, 150 µl of 10% $AlCl_3$ solution was added, and after 6 minutes of reaction, 1 ml of 4% NaOH was added and brought to a total volume of 5 ml with distilled water. Optical density at 510 nm was recorded against a blank and flavonoid concentration estimation was done according to a calibration curve using as standard rutin. Three repetitions of the same variable were made and the average of flavonoid content was expressed as rutin equivalents/fresh weight (ERU mg/g sample).

High-performance liquid chromatography (HPLC). The callus induced on MS variant with 2.4D characterized by the highest flavonoids concentration represented the sample for HPLC.

A Jasco HPLC System (Jasco Europe, Cremella, Itally), equipped with a Nucleosil 100 C18 (Teknokroma) column and coupled with a Fluorescence Detector FP-2020 (Jasco) detector, was used. The elution was performed using a protocol described by Chuanphongpanich et al. (2006). The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B) and methanol (solvent C). The system was run with the following elution gradient program: 0 min, 5% A/95% B; 10 min, 10% A/80% B/10% C; 20 min, 20% A/60% B/20% C and 30 min, 100% A. There was a 10 min post run at initial conditions for equilibration of the column. The flow rate was kept constant at 1 ml/min. The absorbance was monitored in the fluorescence canal, at 280 nm and 320 nm. Prior to HPLC analysis, all solutions were filtered through 0.45 µm membrane filters. Rutin was used as standard solution. A stock standard solution (50 µg/ml) of rutin was prepared in methanol and stored in the dark at 5°C up to three months.

Statistical analysis

The experimental measurements were done in triplicates for each sample. The results are presented as mean values ± standard deviations (Table 1).

For DPPH activity in relation to the total phenolic, flavonoid content and plant segments samples, analysis of covariance (ANCOVA) and Spearman's correlation were conducted using Xlstat addinsoft (Xlstat, 2013 version).

ANCOVA is a technique based on regression and analysis of variance (ANOVA) (Stevens, 1986) and highlights how the dependent variable (DPPH in our case) was influenced by independent variables (total phenolic, flavonoid content as quantitative variables and plant segments as qualitative variables). For both techniques, the results with $p<0.05$ were considered statistically significant.

Spearman's correlation is a nonparametric statistic used as a measure of the power of association between two variables (Hauke and Kossowski, 2011).

RESULTS AND DISCUTIONS

There are many studies concerning the biological roles of secondary metabolites produced by plants through tissue cultures (Çetin et al., 2015).

Since flavonoids were recognized for their health beneficial effects, and the studied species may represent a cheap source (Ghiasy-Oskoee et al., 2018) of metabolites with antimicrobial, cytotoxic, anti-inflammatory and other activities (Al-Snafi, 2016), we have tested the antioxidant activity of various parts of the plant.

We also tried to obtain callus characterized by higher flavonoid content by using *in vitro* cultures. One of the advantages of *in vitro* techniques is represented by reducing the time required to obtain the plant biomass.

Table 1. The antioxidant activity, total phenolic content and flavonoids concentration of different parts of *in vivo* plant and *in vitro* callus of *C. benedictus*

Samples	**DPPH** (mM Trolox/g sample)	**Total phenolic content** (EAG µg/g sample)	**Flavonoids** (ERU µg/g sample)
Achenes	0.34	356.37 ± 1.4	233.27 ± 2.06
Roots	0.2 ± 0.02	274.525 ± 3 5.49	179.35 ± 3.42
Leaves	3.66 ± 0.6	1,483.75 ± 29.87	234.75 ± 0.56
Stem	1.58 ± 0.08	1,069.3 ± 22.45	409.5 ± 2.08
Callus induced on MS + 2.4D	0.99	802.56 ± 15.033	519.38 ± 0.91
Callus induced on MS + 2.4D+BAP	1.42 ± 0.3	563.42 ± 9.52	367.5 ± 1.09

In DPPH assay, the highest free-radical scavenging capacity was observed in leaves (3.665 ± 0.60 mM Trolox/g fresh sample), followed by stem (1.588 ± 0.08 mM Trolox/g fresh sample) and callus induced on MS supplemented with 2.4D and BAP (1.427 ± 0.3 mM Trolox/g fresh sample) (Table 1).

Analysis of secondary metabolites in different parts of *C. benedictus* plant showed that the highest total phenolic content was registered in leaves followed by stem, achenes and roots, while flavonoids concentration was highest in stem followed by leaves, achenes and roots (Table 1).

The total phenolic and flavonoids content from leaves are 5 times and respectively 1.3 times higher than roots extract**.** Our results are in accordance with Can (2017), who showed that contents of these compounds were 2 times higher in leaf methanolic extract than in root extract of *C. benedictus* cultivated in Aegean region from Turkey. The prevalence of polyphenols in leaves and stems can be explained by the higher involvement in gas exchange of plant aerial parts than roots, polyphenols being detected in the vacuoles of guard cells of different species (Karabourniotis et al., 2001), in epidermal and subepidermal cells of leaves and shoots (Lattanzio et al., 2008). Moreover, polyphenols have protective role against UV radiation due to their screening properties (Pereira et al., 2009) and also due to their involvement in antioxidant system adjustments (Lattanzio et al., 2008), thus in some species they usually accumulate in aerial parts, which are the most exposed to UV light.

In our study, a comparative analysis between *in vitro* callus and parts of the *in vivo* plant, showed that the total phenolic content was higher in leaves (1483.75 ± 29.87 EAG µg/g sample) than *in vitro* callus (802.56 ± 15.03 EAG µg/g sample). In the case of flavonoids, *in vitro* callus induced on the medium with 2.4D was characterized by a higher concentration (519.38 ± 0.91 ERU µg/g sample) than *in vivo* plant part (409.5 ± 2.08 ERU µg/g stem sample). *In vitro* callus had 2 times higher flavonoid concentration than originating inoculum (leaves from mother plant) (Table 1).

The ANCOVA has emphasized that DPPH potential was determined by quantitative variables ($R^2 = 98\%$; $F = 27.40$; $p = 0.01$). The results provided by Type I Sum of Squares of ANCOVA revealed that polyphenols are parameters that have significantly influenced ($F = 162.866$; $p = 0.01$) the antioxidant activity.

Instead, the flavonoid content had a marginal significance ($F = 9.140$, $p = 0.057$) on antioxidant activity. Spearman's correlation ($R = 0.888$; $R^2 = 0.789$; $p = 0.0003$) showed the strong association between DPPH and polyphenols.

The biotechnological approaches were used for numerous species from Asteraceae family for *in vitro* flavonoid production (Bharati and Bansal, 2014). Agarwal and Kamal (2007) observed a higher value of total flavonoid content in *Momordica charantia* callus compared to *in vivo* samples, with the maximum amount accumulated in 6-weeks-old callus. Plant growth factors which promote cell growth by stimulating cell division and elongation like auxins and cytokinins are usually used to induce callus (Coenen and Lomax, 1997). 2.4D has been widely used alone or combined with cytokinin (especially BAP) to stimulate callus induction, to obtain cellular mass and to produce bioactive compounds (Castro et al., 2016).

The efficiency of exogenous 2.4-D has also been reported with other Asteraceae medicinal plants like *Carthamus tinctorius* Linn. (Kumari et al., 2015), *Chrysanthemum morifolium* Ramat. (Swarna et al., 2016). The synergism of 2.4-D and BAP has good results in callus induction at *Helianthus annuus* (Inoka and Dahanayake, 2015). A B5 medium supplemented with BA (0.05 mg/l) and 2.4-D (0.5 mg/l) was the best culture medium for callus production for biomass of milk thistle as a potential source of milk clotting peptidases (Cimino et al., 2006).

In our case, the initiation of callus occurred from the tenth day. In terms of morphology, the calli did not differ, being friable and having a pale green colour. The induction rate was low in the first subculture, more exactly 18.18% on MS added with 2.4-D and 36.36% on MS added with 2.4D and BAP.

The obtained calli were clearly different from the metabolic point of view, callus induced on medium with 2.4-D being highly productive for both flavonoids and polyphenols. This may be explained by the herbicidal effect of 2.4D which induces abiotic stress and plant triggers its defense mechanism by producing more secondary metabolites (Ong et al., 2011). The callus induced on medium with 2.4-D in combination with BAP is distinguished by increased antioxidant activity although it does not possess a rich polyphenolic and flavonoid content (Table 1). This may be possible due to other metabolites which contribute to the total antioxidant activity, being known that this species have a high α-tocoferol content, a potent antioxidant.

In our case, medium supplementation with BAP resulted in a decreased level of polyphenols and flavonoids (Table 1). Our finding is in accordance with results where cytokinins inhibited rutin in callus and in adventitious root culture of *Morus alba* (Lee et al., 2011).

A HPLC was performed to check the presence of rutin in *in vitro* callus induced on MS+2.4D. The rutin peak (retention time = 24.0 minutes, ƛ =320 µm channel) was validated in *in vitro* callus; and also, a lot of different elution peaks can be observed in the chromatograms of *in vitro* callus extract (Figure 1).

Our results showed that *in vitro* techniques represent an efficient way to obtain secondary metabolites as flavonoids and/or polyphenols which may be produced in large quantities by increasing cellular biomass and subsequently exploited in the food, cosmetics, pharmaceutical industry.

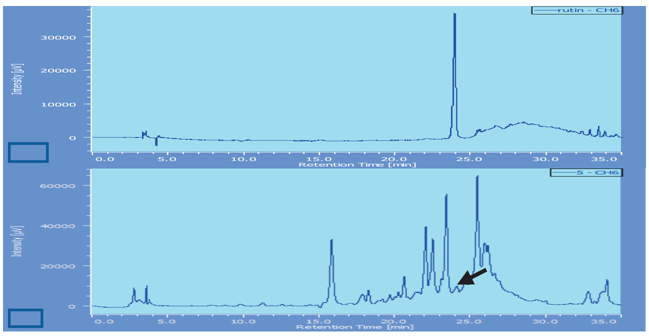

Figure 1. HPLC chromatograms of active principles present in callus sample in 320 µm channel. Arrow indicate the peak correspnding to rutin. A - standard solution, B - callus sample

CONCLUSIONS

In our case, antioxidant activity was correlated with polyphenols content, but there are also other metabolites whose synthesis can be induced *in vitro* and contribute to the total antioxidant activity.

Starting from the same type of explant, two types of callus different from biochemical point of view were obtained in four weeks: one that possesses high antioxidant activity and the second with a rich content of flavonoids and polyphenols.

It was evidenced that *in vitro* induced callus is effective in enhancing the production of flavonoids and polyphenols in a medium supplemented with 2.4-D.

Using HPLC technique, rutin was identified in callus induced through *in vitro* methods; numerous other metabolites were observed.

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PROTEIN HYDROLYSATES: FROM AGRICULTURAL WASTE BIOMASSES TO HIGH ADDED-VALUE PRODUCTS (MINIREVIEW)

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Abstract

The degradation of biomasses originating from agriculture and food processing offers the double benefit of removing polluting waste and introducing new bio-derived products into the market. Protein Hydrolysates (PHs), used as sources of bioactive peptides and amino acids play a fundamental role among biotechnological products deriving from waste biomasses. PHs are bio-based chemicals with high added value that can be produced through different types of hydrolysis: chemical, microbiological, enzymatic or mixed. Depending on the biomass used and the hydrolysis procedure chosen, products endowed with different features, such as high biostimulating, hormonal, fertilizing and nutritive capacities, can be obtained. The production of PHs from vegetable biomasses prove pivotal, as they offer absolute health guarantees and can be marketed in areas that do not abide products derived from animal waste, which currently are often found in the market. Here, the various possible applications of PHs will be discussed, along with the different processes for their production starting from agro-food waste biomasses, paying particular attention to the advantages of enzymatic hydrolysis.

***Key words**: Protein Hydrolysates, Waste Biomasses, Circular Economy, Chemical Hydrolysis, Enzymatic Hydrolysis.*

INTRODUCTION

Industrial processing of agricultural and food products originates large amounts of by-products which need to be disposed of. Although the successful application of the principles of green chemistry is fundamental to reduce the production of waste substances, it is not possible to avoid their generation. Considerable effort is currently going into finding employment and usefulness for the inevitably produced waste, with the aim being to give new value to this waste biomass by converting it into chemicals that can be used in other areas and reintroduced into the market, thus responding to the challenge of Circular Economy (Stahel, 2016; Tuck et al., 2012).
Biomasses can be defined as those organic raw materials, featuring a natural biological origin, which can become substrates for other processes. Depending on its chemical composition, a waste biomass can be classified into four categories (Tuck et al., 2012): polysaccharides, lignin, triglycerides (from fats and oils) and proteins. The large amount of by-products deriving from industrial processing can thus be exploited to make compost, biogas or other low-added value products. Further to this, waste biomasses are also especially interesting for their possible conversion into high-added value hydrolysates (Martínez-Alvarez et al., 2015).
In fact, different types of hydrolysates can be produced, with concentrations in carbohydrates, proteins and lipids that are variable depending on the origin of the initial biomass. In particular, a considerable amount of biomass possesses high protein content, immediately appealing from a nutritional and physiological point of view. The production of food and drinks is the sector most involved in this respect: by-products of the sugar and ethanol industry (*i.e.* vinasse from sugarcane or sugar beet), maize fermentation waste

(Distiller's Dried Grains with Soluble), oil seed press cake, fish silage, tea and coffee grounds and agricultural wastes coming from different crops, are all examples of waste containing a large quantity of proteins (Tuck et al., 2012).

In order to obtain Protein Hydrolysates (PHs) from a biomass, the latter can undergo treatments of different types ranging from chemical (using acids or alkalis), to microbiological and enzymatic, or mixed.

Hydrolysis has traditionally been carried out with extremely low or high pH chemicals, at high temperatures, and, only more recently, through microorganisms or enzymes (Diguta et al., 2007; Bruni et al., 2010) both in their free and immobilized form. The disadvantage of the first approach is the possible development of toxic side-products, whereas the high level of energy required for a purely temperature-based process is negative in terms of costs and carbon dioxide budget. The production of PHs by the enzymatic route instead exploits the ability of these biomolecules to work under mild conditions of temperatures and pH, avoiding the production of toxic contaminants in the final product (Abd et al., 2017).

By performing hydrolysis, the protein fraction of the waste biomass can be broken down into the amino acids that make it up. PHs are produced starting from the hydrolysis of intact proteins, with the formation, by breaking the peptide bonds, of a mix of small peptides of different sizes and free amino acids. They have various applications: PHs can be incorporated in other substances, or used directly as pharmaceutical, cosmetic and nutritional products. It is important that the hydrolysis product is stable in terms of oxidation and has good organoleptic characteristics. For example, if the hydrolysate has to be ingested, it is essential for it to possess a high content of nutrients and to be easily digestible and bioavailable (Canistro et al., 2017; Li-Chan, 2015; Martínez-Alvarez et al., 2015).

The properties of the PHs, and therefore their application, also depend on the degree of hydrolysis of the proteins, that is the number of peptide bonds cleaved, divided by the total number of peptide bonds and multiplied by 100 (Pasupuleti et al., 2008): the average hydrolysed proteins are used for example in sports and clinical nutrition, while the highly hydrolysed ones, thanks to their hypoallergenic nature, are used in the formulation of products for childhood, as alternatives to cow's milk proteins (Kiewiet et al., 2018).

In particular, because of the increasing interest in plant derived products, agricultural wastes have recently aroused considerable attention for the production of PHs. The preference for non-animal derived products is also due to the lack of prion-risk, and consequently because there is no need for strong final chemical treatments. Moreover, it has been observed that these hydrolysates can also be sources of bioactive peptides, with possible clinical applications such as antithrombotic, antihypertensive, antimicrobial, anti-carcinogenic, antioxidant and immune-modulating agents (Kiewiet et al., 2018).

Here we report an overview on a series of possible applications for PHs originated from agri-food waste biomasses. Later, we will discuss the differences between chemical hydrolysis and microbiological or enzymatic catalysis, and specifically on the advantages of using the latter approaches over the common chemical route.

APPLICATIONS

Many are the potential applications of plant-derived PHs. They can serve a major role in animal nutrition for their straightforward nutritional, physiological and regulatory functions, but they can also be used as biofertilizers, in that hydrolysates are able to improve nutrient assimilation of crops and to mitigate crop stress (Halpern et al., 2015). Moreover, those hydrolysates which carry out biological functions other than mere nutritional ones are known as bioactive peptides, *i.e.* those able to act as antihypertensive, antioxidant and anti-inflammatory agents (Hou et al., 2017; Zou et al., 2020).

As for their nutritional functions, PHs boost feed efficiency and can promote growth rate. The hypoallergenic nature of hydrolysates makes them also suitable ingredients for infant food formulations or as supplement in the diets of children suffering from severe food allergies (Schaafsma, 2009). In addition, PHs exhibit interesting rheological properties, like better solubility than intact proteins, and they can be endowed with peculiar physicochemical and functional features for specific applications (Xia et. al., 2012). As an example, rice PHs are employed both as nutritional supplements, and as flavour enhancers in food and as whiteners for coffee. (Fabian & Ju, 2011; Phongthai et al., 2017). To date, not only offers the market hypoallergenic formulations for children, containing widely hydrolysed proteins, but also, PHs are used in the nutrition of sick subjects, as they are easier to digest than intact proteins. Finally, in the nutrition of athletes, PHs are used as sport beverages for recovery after training, in order to reduce inflammation and muscle pain.

An excellent source of PHs is represented by soybean waste. In fact, soybean meals comprise anti-nutritional substances and protein-type allergens, while fermented soybean leads to PHs which can be efficiently used to improve growth-performances in the diet of weaning pigs, young calves, poultry and fish, as well as companion animals (Hou et al., 2017). On the other hand, plant-derived hydrolysates rich in glutamine and glutamate (*i.e.* wheat gluten) can be added to the diet of companion animals to provide savoury flavours, thus enhancing the palatability of the feeding products (Nagodawithana et al., 2010). Broadly speaking, a noticeable health improvement for companion animals can be achieved through the addition of PHs to their diet, especially resulting in good gut health and general well-being (Hou et al., 2017).

A further application of PHs is the one as fertilizer, thanks to their ability to improve nutrient assimilation and to reduce crop stress. PHs are indeed able to guarantee higher yields and productivities in the case of different crops, improving the plant nutritional balance and increasing its endogenous defences against abiotic stresses (like thermal shocks and lack of water). For example, a biostimulant effect on yield performance and nutritional quality has been demonstrated for greenhouse tomato (Colla et al., 2017) treated with 3 $mL \cdot L^{-1}$ legume-derived PHs. As is the case with soybean PHs, the rate of nitrogen, phosphorus and potassium (NPK) release, a key factor for crop growth, can be improved with the addition of hydrolysates in the fertilizer composition, leading to increased crop yields and enhancing the activity of microorganisms in the soil (Liu et al., 2019).

Further to this, PHs can be exploited for their antihypertensive, antioxidant and anti-inflammatory properties as well as for their positive effects on the immune system. If this is the case, hydrolysates with biological functions are called bioactive peptides.

PHs with an immunomodulating role could slow down or prevent the onset of a series of immune-correlated diseases. Lately, progress has been made in defining which characteristics are actually useful for an optimal and reproducible positive effect on the Immune System (IS). A development in this field could improve the effectiveness of existing products that contain hydrolysates, with applications in sports nutrition, clinical nutrition and infant formulas. In this respect, the lymphocytic proliferation and the phagocytic capacity of macrophages are both crucial points to be reckoned with. The increase in lymphocytic proliferation has been observed after stimulation with different types of PHs, such as soy and wheat, but not all hydrolysates necessarily stimulate this proliferation. The effects on phagocytosis also depend on the protein source that is used, for example soy and wheat again promote it, while the hydrolysed rice protein inhibits the phagocytic activity of macrophages (Kiewiet et al., 2018).

In addition, some hydrolysates also have anti-inflammatory properties. This characteristic is mainly attributed to those hydrolysates, *e.g.* rice bran PHs, which involve the reduction of the intestinal damage caused by the inflammatory condition, through a decrease in the production of pro-inflammatory cytokines (Boonloh et al., 2017).

The clinical condition of hypertension is responsible for 45-51% of the total deaths in the modern world. High blood pressure can lead to the development of serious cardiovascular diseases, which can affect the heart, blood vessels and kidneys. The renin-angiotensin-aldosterone system (RAAS) is the one most involved in the control of blood pressure: the renin is synthesized in the kidneys and released into the bloodstream, where it goes to cleave the N-terminal region of angiotensinogen and produces a decapeptide, angiotensin-I, which circulates in the blood until its C-terminal residue is also cleaved by Angiotensin-I Converting Enzyme (ACE), with the formation of an 8 amino acid peptide, called Angiotensin-II, which is a powerful vasoconstrictor. Current hypertension medications (Captopril, Enalapril and Lisinopril) are based on ACE inhibition, but the need to reduce the side effects of nausea, vomiting and dry cough is pushing researchers to find natural alternatives, that is to say peptides deriving from the hydrolysis of food proteins, such as hemp seed or wheat bran.

The Hemp Seed Proteins (HSPs) can be obtained during the production of an edible oil: starting from the Hemp seed Protein Meal (HPM), which has a protein content of 37%, HSPs can be isolated for the preparation of a PH through the use of different proteolytic enzymes. By testing the *in vitro* effects of HSP hydrolysates, it was noted that these act by inhibiting the renin and ACE activity, thus preventing blood pressure from rising (Malomo et al., 2015).

Another example of antihypertensive bioactive peptide is represented by the Wheat bran PH (WPH). Wheat bran, generated as a by-product of the wheat industry, mainly consists of the seed coat resulting from the transformation of wheat into flour (Coda et al., 2014). As of today, most wheat bran is used as a low-value component for animal feed and human consumption. The WPH can instead be seen as a high-value added product with significant biological activity. In particular, highly hydrolysed WP (peptide fraction < 1 kDa) were found to have high antihypertensive efficiency (Zou et al., 2019), supposedly because these small peptides can either be better absorbed in the intestine or show increased ability to interact and thus inhibit the involved enzymes (ACE and renin). Moreover, this WPH fraction also exhibits high antioxidant activity against oxygen radicals, which is another relevant feature to be pursued.

This body of evidence gives information on how the PHs obtained from both hemp seeds and wheat bran can be used effectively for the formulation of functional foods and nutraceuticals with antihypertensive activities.

Lastly, PHs have recently been tested as promoters of non-haem iron absorption. Iron is one of the trace elements most present in the human body and its deficiency, in addition to leading to anaemia, can also decrease productivity at work, impair physical capacity and reduce endurance in sports. In this view, the ability of PHs to form ferrous chelates, *i.e.* molecules linked to ferrous (Fe^{2+}) ions, is a desirable quality to promote iron absorption, thus preventing and reducing fatigue (Li et al., 2017). Fatigue is a complex physiological and biochemical process, which represents a widespread social problem common to those people who, due to strong competition in the workplace or irregular lifestyle, are subject to enormous pressure. Mineral-based products against fatigue are therefore a developing field (Huang et al., 2015).

For these reasons, PHs able to chelate iron and other metals, thus enhancing their absorption, have been arousing increasing interest. PHs can indeed be exploited to keep iron soluble, reduce ferric iron to ferrous iron and promote transport across cell membrane, especially into the gut. In this respect, barley, chickpea, rice and soybean PHs can all be listed among mineral chelating peptides (Cao et al., 2007; Eckert et al., 2016; Li et al., 2017; Lv et al., 2009; Lv et al., 2013; Torres-Fuentes et al., 2012; Wakabayashi et al., 1989; Zhang et al., 2014).

CHEMICAL HYDROLYSIS

Until recently, the production of PHs was mainly carried out chemically, employing acid or basic hydrolysis at high temperatures. However, these types of processes are difficult to control and above all lead to the formation of poor quality products due to the loss of assimilable amino acids and the production of modified amino acids (Tavano, 2013). Acid hydrolysis is usually carried out by using 6 M HCl at 110°C for more than 24 hours leading to the degradation of some amino acids, namely arginine and tryptophan, and causes the formation of unwanted secondary compounds such as chlorides (Corte et al., 2014; Tsugita & Scheffler, 1982).

Similarly, alkaline hydrolysis, conducted with strong bases, namely NaOH or KOH, causes the degradation of amino acids such as cysteine, arginine, threonine, serine, and isoleucine and also leads to the formation of modified amino acids such as lysinoalanine and lanthionine (Fountoulakis & Lahm, 1998; Tavano, 2013). Furthermore, the high content of acid or basic residues in the final hydrolysates limits their applicability especially in the agricultural sector and in the food industry (Chervan & Deeslie, 1984).

Table 1. Functions of agri-food waste biomass hydrolysates listed by area of application

Area of application	Biomass	Function	Ref.
Nutrition	Rice bran	Flavour enhancers in food Whiteners for coffee	Fabian & Ju, 2011; Phongthai et al., 2017
	Soybean	Diet supplements to improve growth-performances	Hou et al., 2017
	Wheat Gluten	Palatability enhancers	Nagodawithana et al., 2008
Farming	Vegetables	Biostimulant effect on yield performance and nutritional quality of greenhouse tomato	Colla et al., 2017
	Soybean	Increasing crop yields and enhancing the activity of microorganisms in the soil	Liu et al. 2019
Medicine	Soybean and Wheat	Increasing lymphocytic proliferation and phagocytic capacity of macrophages	Kiewiet et al., 2018
	Rice bran	Anti-inflammatory properties by decreasing pro-inflammatory cytokines	Boonloh et al., 2017
	Hemp seed	Antihypertensive power by inhibiting the renin and ACE activity	Malomo et al., 2015
	Wheat bran	Antihypertensive power by inhibiting the renin and ACE activity	Zou et al., 2019
		Antioxidant activity against oxygen radicals	
	Barley	Promoters of non-haem iron absorption	Eckert et al., 2016
	Chickpea	Promoters of non-haem iron absorption	Torres-Fuentes et al., 2012
	Rice	Promoters of non-haem iron absorption	Cao et al., 2007
	Soybean	Promoters of non-haem iron absorption	Lv et al., 2009; Lv et al., 2013; Wakabayashi et al., 1989; Zhang et al., 2014

The chemical hydrolysis obtained with both acid and basic agents is therefore an extremely aggressive process, which, on the one hand, allows a high percentage of free amino acids to be obtained, but on the other hand, puts them through structural changes making them no longer assimilable.

One of the methods for determining the quality of the hydrolysate is to evaluate the degree of racemization, which is the phenomenon that causes the passage of amino acids from their levogyrous form, the biologically active form, to a dextrogyrous one. The latter represents the most abundant form in the PHs obtained chemically as the aggressiveness of the process modifies the natural form of the amino acids. A high degree of racemization therefore represents a negative quality index, since the right-handed amino acids cannot be assimilated by either animal or vegetable living organisms (Rikken & Raupach, 2000).

MICROBIAL HYDROLYSIS

Another viable route to the preparation of PHs is based on the use microorganisms, which, through the action of specific enzymes such as proteases, lead to the formation of small peptides and free amino acids. Microorganisms are endowed with a whole series of enzymatic kits that enable the degradation of organic matter, not only of protein origin but also polysaccharides and lipids (Hou et al., 2017; Smid & Lacroix, 2013).

The microbiological hydrolysis of the biomass protein component is divided into liquid or solid depending on the percentage of humidity of the substrate used; as a rule, what varies is the microbial flora involved.

One of the first products obtained through microorganism-mediated hydrolysis was soy sauce (Hou et al., 2017; Pasupuleti, et al., 2008). Microbiological hydrolysis is now widely used to obtain hydrolysates starting from plant biomasses (Bah et al., 2016; Li-Chan, 2015; Lòpez-Barrios et al., 2014) and in the dairy industry (Hou et al., 2017).

A series of clear advantages comes with the employment of microorganisms to produce PHs. This is mainly linked to the fact that by not resorting to aggressive chemical agents and high temperatures, the formation of

biologically active small peptides and free amino acids can be secured; moreover, it has been shown how microorganisms can also remove hypoallergenic or anti-nutritional substances from the final products.
However, even microbiological hydrolysis suffers some disadvantages, expressly due to the high production costs, as well as to the susceptibility of microorganism activity to changes in the environmental conditions (Hou et al., 2017).

ENZYMATIC HYDROLYSIS

PHs can also be obtained enzymatically by resorting to purified enzymes in their free or immobilized form. In this case, the hydrolysis process is again more advantageous than the chemical one, since it is carried out under mild conditions of temperature (40-60°C) and pH (6-8), resulting extremely favourable from an economic and environmental point of view.
Enzymatic hydrolysis does not lead to the formation of unwanted and often toxic secondary products and, just like microbial hydrolysis, preserves the structure of the amino acids, which maintain their biologically active levogyrous form (Clemente et al., 1999; Clemente et al., 2000). The hydrolysates obtained through the enzymatic approach are also soluble, more resistant to heat and more resistant to precipitation (Clemente et al., 2000; Fox et al., 1982).
The most preferred enzymes for the preparation of PHs are proteases of microbial origin, composed of exopeptidases and endopeptidases according to the type of catalytic reaction performed; in fact, the exopeptidases make cuts at the level of the terminal region of the protein, while endopeptidases give internal cuts (Hou et al., 2017; Wu et al., 2013).
The enzymes involved in the production of PHs can derive from animal, vegetable or microbial sources. Pancreatin, trypsin, and pepsin are the main proteolytic enzymes obtained from animal sources, while papain and bromelain are extracted from plant organisms. Bacteria and fungi, however, represent the largest source of proteolytic enzymes, as they release enzymes directly into the extracellular environment, making the latter easier to extract. Moreover, proteases of bacterial and fungal origin exhibit a large range of optimal temperatures and pH (Dixon, 1979; Hou et al., 2017; Kunst, 2003).
The properties and composition of PHs obtained through enzymatic hydrolysis are crucial for their positioning in the market as commercial products. For example, the PHs produced enzymatically from perilla seed meal (PSM), a by-product of the production of perilla seed oil, were found to have a very high content of essential amino acids (*e.g.* lysine). Additionally, PSM hydrolysates feature better functionality relative to PSM protein isolates, as the former exhibit higher solubility, greater oil absorption capacity, and superior emulsifying and foaming properties (Park & Yoon, 2019). Another example is the partial hydrolysis of quinoa protein (QP) isolates, by means of a fungal serin peptidase, for the production of QP hydrolysates to be used as food additives in semi-solid healthy foods with both antioxidant activity and gelling capability (Galante et al., 2020). Again, Maqsoudlou et al., 2019 proposed the hydrolysis of pollen using pepsin and trypsin under controlled conditions for the generation of peptides possessing different sizes. A heterogeneous composition was revealed in the peptide sequences, with glycine and alanine being the two most abundant hydrophobic amino acids. Pollen PHs also possess significant bioactivities, namely antioxidant and ACE inhibitory activities, which make them promising functional ingredients in food formulations.
However, although enzymatic hydrolysis is extremely advantageous for the low environmental impact, thanks to the mild reaction conditions required, on the other hand, it is very expensive due to the need for the enzymes to be purified. Once the final hydrolysate is obtained, further purification steps are also required when free enzymes are employed, as well as deactivation processes so that the enzyme does not alter the desired products. Indeed, it is necessary to use high temperatures to inactivate proteolytic enzymes, but at the same time, these stringent conditions cause the irreversible denaturation of the enzymes, making their recycling impossible (Rocha et al., 2011; Huang et al., 1999).
In order to overcome these drawbacks, enzymes immobilized on inert substrates (*e.g.*

films, column reactors, nanoparticles) have recently been developed. By doing so, enzymes maintain their catalytic capacity and their stability under wide reaction conditions, but at the same time they become easily separable from the reaction product through sedimentation, filtering or centrifugation.

There are many examples in which PHs have been prepared through the use of immobilized enzymes. For example, active corn peptides were obtained from zein by alcalase and trypsin co-immobilized on a calcium alginate-chitosan composite carrier (Wang et al., 2014). In a study by Wei et al., 2018, immobilized alcalase and flavourzyme were instead used to prepare flaxseed PHs capable of improving the flavour in Maillard reaction products. More recently, alkaline proteinase was covalently immobilized on amino-functionalized magnetic nanoparticles, resulting in better storage stability compared to free enzyme, for soy protein hydrolysis (Zhu et al., 2019).

CONCLUSIONS

In a society where the search for new sources of energy is becoming more and more vital, waste biomasses cannot be seen as something to be disposed of, but instead as new resources to take advantage of.

Agriculture and food industry generate large amount of waste biomasses, rich in protein content, so that they can be exploited for the production of PHs to be reintroduced into the market, thus enjoying the ride of Circular Economy.

Further to this, in view of their many applications ranging from nutritional to biological and clinical, PHs have been awarded the deserved title of high added-value products. Here we have highlighted a long list of effective practical applications for PHs derived from agri-food waste biomasses, unravelling their endless potential.

In addition, we discussed the key strength of microbiological and enzymatic catalysis, which allow mild reaction conditions with respect to harsh chemical hydrolysis. Finally, we brought into clear focus the possibility to resort to immobilized enzymes in order to carry out protein hydrolysis: this is an ever-growing strategy which has been gaining a position of special prominence, as it combines the eco-friendliness of the enzymatic approach with the economic advantage of reusing immobilized enzymes.

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EFFECTS OF CORN DISTILLERS DRIED GRAINS WITH SOLUBLES ON REPRODUCTIVE PERFORMANCE OF ARBOR ACRES BROILER BREEDER HENS

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Abstract

The objective of this study was to examine the productive performance, egg quality and reproductive parameters of commercial Arbor Acres (AA) broiler breeder hens fed different levels of corn distillers dried grains with solubles (DDGS). A total of 2340 AA broiler breeder hens, 26 weeks of age, were allocated for a 14-week experimental period in a feeding trial consisting of 3 dietary treatments (0, 10, and 15% DDGS), and 4 replicates per treatment. Each replicate consisted of a group of 195 hens (male to female ratio 1: 10). All experimental diets were prepared as iso-protein (16%) and isocaloric (ME 11.7 MJ/kg) and with similar content of total sulfur amino acids, Ca and available P. The use of DDGS up to 15% in AA broiler breeder hens diet did not have adverse effects on egg production, feed intake, feed conversion ratio and egg mass (P>0.05). DDGS, also led to a significantly increased yolk color intensity (P<0.001), while having no effect on egg interior and exterior quality, especially on eggshell quality, such as egg specific gravity. Fertility and hatchability of fertile eggs were not affected by the dietary DDGS levels. Based on the results, DDGS could be included up to a level of 15% in AA broiler breeder hens' diet without any significant detrimental effects on the egg production, egg quality and reproductive performance.

Key words: *Arbor Acres broiler breeder hen, distillers dried grains with solubles, egg quality, hatchability.*

INTRODUCTION

Broiler breeder diets influence subsequent egg production performance and also embryogenesis and hatchability of broiler eggs (Adeyemo et al., 2007; Bozkurt et al., 2008; Javanka et al., 2010; Krawczyk et al., 2012). Efficient management practices must be maintained for the female broiler breeder to lay eggs and therefore pass on her genetic potential for rapid growth to her progeny (Richards et al., 2010). Therefore, one of the challenges of a nutritionist should be to include in broiler breeder diets of any *alternative* product as an ingredient to minimize the cost of feed. Distillers dried grains with solubles (DDGS) production has increased greatly over the past several years, mostly because of the increase in beverage and as well as ethanol production. DDGS as a by-product has traditionally been fed primarily to ruminants due to its high fiber content and variability of nutrients (Singh et al., 2005). Poultry diets must be formulated precisely, and previous research has shown that DDGS is a valuable source of energy, protein, and amino acids (Parsons and Baker, 1983; Robertson et al., 2005; Wang et al., 2007). Likewise, DDGS are a good source of phosphorus (P), containing 0.72% total P (NRC, 1994), and the bioavailability of P is higher than the 25 to 35% that is typical of most plant ingredients.

In the literature, there is an abundance of information about the composition and use of DDGS presented in various forms. In many earlier studies, it was concluded that DDGS was a useful feed ingredient for laying hens, broiler chickens and turkeys (Roberson, 2003; Noll et al., 2004; Roberson et al., 2005; Lumpkins et al., 2004; 2005; Noll and Brannon, 2006; Świątkiewicz and Koreleski, 2006; 2008; Loar et al., 2010; Masa'deh et al., 2011; Shim et al., 2011; Niemiec et al., 2012; 2013). There is sparse research regarding the use of DDGS in broiler breeder hen diets (Krawczyk et al., 2012; Mejia et al., 2012).

Feeding higher levels of this by-product could have a significant effect on feed costs for poultry producers as a result of the higher availability of this by-product for livestock usage and the current price fluctuations of feed ingredients.
The current study was designed to assess the effect of the inclusion of DDGS in the maternal diet on embryo viability, hatchability, and initial growth of the progeny. An important aspect of the study was to evaluate how this ingredient affects the egg quality and, in particular, egg interior and exterior quality. Therefore, we assessed the egg production, quality of eggs and reproductive parameters during peak production on Arbor Acres broiler breeder hens that were fed diets with graded levels of DDGS, providing the opportunity to replace corn and soybean meal.

MATERIALS AND METHODS

The study protocol was approved by the Animal Ethics Committee of the National Research and Development Institute for Animal Biology and Nutrition Baloteşti, Romania, and was in accordance with the principles of the EU Directive 2010/63/EU and Romanian Law on Animal Protection.

Birds, management and diets

A total of 2340 broiler breeder hens Arbor Acres, 26-wk-old, were weighed individually (BW = 2.90 ± 4.7 kg) and reared in 12-floor pens with wood-shaving litter (6.2 birds/m^2). All birds were housed under controlled climate conditions (temperature 18 ± 2^0C, relative humidity 70%, light schedule 16L: 8D). Feed and water were provided *ad libitum* throughout the experimental period. Vaccination and medical care were done according to common veterinary care under veterinarian's supervision.
Birds were randomly allocated to 3 dietary treatments of 780 hens per treatment (male to female ratio 1: 10). Each dietary treatment had 4 replications. Experimental diets were fed from 26 to 39 wk of age. Males were fed together with hens in the same feeders; separate-sex feeding was not applied. So, the amount of daily feed allowance for males was assumed to be similar to females. Corn-DDGS was included in the breeder's diet at 0, 10, and 15%, respectively (DDGS-0, DDGS-10 and DDGS-15) at the expense of corn and soybean meal. Diets were in mash form and were manufactured to be isonitrogenous, isocaloric, and with similar content of total sulfur amino acids (TSAA), calcium and available P compared to breeders' recommendation (Arbor Acres, 2013).

Sampling and analysis

To ensure the accurate formulation of the experimental diets (Table 1), samples of ingredients were analyzed using standard procedures according to the methods of the Commission Regulation (EC) no. 152 (OJEU, 2009). The metabolizable energy content of the diets was calculated on the basis of the energy content of individual feed ingredients using regression equations from the NRC (1994). Amino acids (AA), excluding tryptophan, which was not determined, were analysed using a high performance liquid chromatography (HPLC system; Thermo Fisher Scientific Inc., San Jose, CA USA), fitted with a quaternary system for solvent pumping, and with photodiode array detectors (Surveyor PDA Plus Detector), according to the conditions described by Ciurescu et al. (2018). We used a highly pure Hypersil GOLD silica chromatographic column, designs to optimize separations and maximize productivity. AA standards certified and purchased from Sigma-Aldrich were injected for the qualitative and quantitative determinations. All reagents were certified and had HPLC purity.

Egg production and performance

Daily hen egg production and mortality were recorded daily throughout the 14-week experimental period. Egg characteristics were measured daily for all eggs laid during week 39 and included egg and egg components weight (albumen [AW], yolk [YW] and eggshell [ESW]), eggshell specific gravity (ESG), albumen height (AH) and yolk color. ESG was assessed by the flotation method (Bennett, 1992). Eggs were then weighed and cracked carefully separating contents from eggshell, using a micrometer with ultrasonic wave system (SANOVO Technology A/S, Odense NV, Denmark). The same equipment also detected the AH and the yolk color with an RGB sensor.

Table 1. Composition of experimental diets (g/kg^{-1})

Ingredients	DDGS-0	DDGS-10	DDGS-15
Corn	433.2	353.6	319.3
Wheat	270.0	302.4	313.7
Soybean meal	195.0	143.0	117.3
Corn-DDGS	-	100.0	150.0
Vegetable oil	4.0	5.7	6.0
Monocalcium phosphate	11.0	8.0	6.0
Calcium carbonate	73.5	74.2	74.7
Sodium chloride	2.0	2.0	2.0
Vitamin-mineral premix[1]	10.0	10.0	10.0
DL-methionine (99%)	1.3	1.1	1.0
Calculated nutrient composition (g/kg)			
Crude protein	160.0	160.3	160.4
ME (MJ/kg)	11.7	11.7	11.7
Lysine, total	7.7	7.1	6.8
Lysine, digestible	6.7	6.0	5.7
Methionine, total	3.8	3.8	3.8
Methionine, digestible	3.6	3.5	3.5
TSAA, total	6.7	6.7	6.7
TSAA, digestible	6.0	5.8	5.7
Calcium	30.0	30.0	30.0
Phosphorus, available	3.4	3.4	3.4
Crude fat	30.0	37.4	40.6
Crude fibre	24.0	29.0	31.5
Analysed (g/kg)			
Dry matter	898.9	902.5	899.3
Crude protein	159.6	161.2	159.8
Crude fibre	28.5	27.6	29.9
Crude fat	29.2	38.5	45.7

[1]Supplied per kg diet: vitamin A from retinyl acetate, 12,000 IU; vitamin D_3, 3,500 IU; vitamin E from dl-α-tocopheryl acetate, 100 mg; menadione sodium bisulphite, 5 mg; thiamine mononitrate, 3 mg; riboflavin, 12 mg; vitamin B_6 from pyridoxine mononitrate, 5 mg; cyanocobalamin, 0.030 mg; Ca-panthotenate, 13 mg; niacin, 50 mg; folic acid, 2 mg; d-biotin, 0.3; choline (choline chloride), 1200 mg; Mn, 120 mg; Zn, 110 mg; Fe, 50 mg; Cu, 10 mg; I, 2.0 mg; Se, 0.30 mg; Co, 0.25 mg.

Eggshell weight (ESW) was expressed as a percentage of the egg weight. Albumen weight (AW) was calculated by subtracting the weights of yolk (WY) and eggshell from the weight of the egg. Egg mass was calculated by multiplying egg weight by egg production.

At 39 week the residual feeds and birds were weighed and the final BW, feed intake, and feed conversion ratio (FCR) were determinate. FCR was expressed as kg of feed consumed per kg of egg produced. The magnitude of production variables such as feed intake and egg production was adjusted for hen mortalities. Deaths were recorded daily as they occurred. Replicate pen means for each parameter were used for analyses.

Hatchability

Hatching eggs were collected daily at 39 weeks of age (n = 1,300), identified by date of collection and pen, and were stored at 18^0C and 70% relative humidity until incubation. Any cracked eggs or eggs weighing less than 52 g were not used in this experiment. The remaining eggs were incubated in a Petersime Incubator (Petersime Incubator Co., Ohio, USA). Incubator was set at 37.6^0C dry bulb and 28.6^0C wet bulb temperatures. Eggs were candled on day 10 of incubation for determination of infertile eggs. All infertile eggs were opened and examined macroscopically for evidence of embryonic mortality. Fertility was expressed as a rate of fertile eggs to total eggs set. On day 19, eggs were transferred to the hatching cabinet of the same incubator for hatching. Hatching incubator was set at 37.5^0C dry bulb and 29.2^0C wet bulb temperatures. The number of eggs that hatched was recorded at 21.5 days of incubation. Hatchability of fertile eggs was expressed as the rate of hatching chicks to fertile eggs. After hatching, the initial chick body weight was determined.

Statistical analysis

The collected data were analyzed by the General Linear Models (GLM) procedure using the SPSS software version 20.0 (SPSS, 2011). *Post hoc* Tukey's multiple comparison test was used to evaluate the statistical significance of differences among the treatment means. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSIONS

Nutrient composition of corn-DDGS

Table 2 shows the chemical composition of corn DDGS (n = 28 samples). When converted to a dry matter basis, the contents of crude protein ranged from 24.8 to 36.2%, ether extract from 10.8 to 12.5%, crude fiber from 9.1 to 10.5%, ash from 5.0 to 5.7%, and starch from 4.1 to 13.3%. The coefficient of variation (CV) for these nutrients ranged from 4.3 to 45.7%. The average values for crude protein, ether extract, fiber, ash, and starch were 25.1, 11.8, 9.4, 5.2, and 8.3%, respectively. The average dry matter content was 93.6% with a CV of 11.9%.

In an early study, Cromwell et al. (1993) evaluated the physical, chemical, and nutritional properties of DDGS from nine different sources (two from beverage and seven from fuel-alcohol production systems), and found that considerable variability in nutrient contents existed among DDGS samples. A decade later, Spiehs et al. (2002) evaluated the nutrient content and variability of DDGS in a total of 118 samples from 10 fuel-ethanol plants during 1997, 1998, and 1999. They noted that the values are not substantially different from those of Cromwell et al. (1993), and both showed higher variation in ash content and lower variation in dry matter content. Belyea et al. (2004) analyzed 235 DDGS samples from a fuel-ethanol plant in Minnesota and found that the average values (% dry matter) for protein, oil, ash, crude fiber, and ADF were 31.4, 12.0, 4.6, 10.2, and 16.8, respectively. They also reported the average content of residual starch as 5.3%. Thus, Belyea et al. (2004) gave higher average values of protein, oil, and crude fiber and a lower value of ash compared to Spiehs et al. (2002). Liu (2008) showed average values of six DDGS samples for protein, oil, ash, and starch as 27.4, 11.7, 4.4, and 4.9%, respectively, dry matter basis. The lower estimate of the protein value as compared with the previous three studies might be due to the use of 5.75 as conversion factor from nitrogen instead of 6.25. We also assessed amino acids (AA) content on 12 corn-DDGS samples, and the average content of lysine varied from 6.67 to 8.05 g/kg (converted to dry matter basis), methionine from 5.66 to 7.10 g/kg, threonine from 8.55 to 9.82 g/kg and arginine from 8.10 to 11.36 g/kg (Table 2).

Table 2. Chemical composition of the DDGS[1] (g/kg DM)

Nutrients	Mean	Limits	CV[2]
n	*28*	*28*	
Dry matter	936	925-945	11.9
Crude protein	251	248-362	4.3
Ether extractives	118	108-125	5.8
Crude fibre	94	91-105	10.1
Ash	52	50-57	7.2
Starch	83	41-133	45.7
ME[3] (kcal/kg)	2.464	2.350-2.562	136.5
n	*12*	*12*	
Amino acids (g/kg DM)			
Lysine	7.37	6.67-8.05	12.8
Methionine	6.20	5.66-7.10	5.7
Cysteine	4.59	4.23-5.44	11.4
Threonine	9.08	8.55-9.82	7.3
Tryptophan	-	-	-
Leucine	21.69	19.86-24.77	16.5
Arginine	9.75	8.10-11.36	28.7
Histidine	6.52	6.33-8.94	11.8
Isoleucine	9.94	8.85-12.10	8.9
Valine	13.59	12.76-17.55	12.6
Phenylalanine	12.97	12.54-13.33	4.1
Tyrosine	7.71	5.37-9.21	38.7
Aspartic acid	20.85	20.66-21.33	6.9
Serine	16.22	16.11-16.53	4.2
Glutamic acid	41.63	41.61-41.74	5.7
Proline	25.77	25.70-25.81	6.6
Glycine	11.14	11.11-11.22	3.3

[1]Analysed data in INCDBNA chemistry laboratory; [2]Coefficient of variation, (%); [3]Metabolizable Energy value was calculated based on regression equations from the NRC (1994).

Lysine was the most variable among the 18 both essential and nonessential measured, with a CV = 12.8%, followed by leucine with a CV=16.5%, arginine with a CV = 28.7%, and tyrosine with a CV =3 8.7%. Nevertheless, lysine, one of the essential amino acids, was contained 2.9 times higher in DDGS than that of corn. In the Spiehs et al. (2002) study, 118 DDGS samples were analyzed for 10 essential amino acids; lysine was found to be the most variable among the 10 amino acids measured, with an average CV = 17.3%. Cromwell et al. (1993) and Spiehs et al. (2002) only analysed essential AA, but others (Batal and Dale, 2006; Han and Liu, 2010) looked at content of both essential and nonessential Salim et al. (2010) noted that although the nutrient content of DDGS is relatively consistent within the same processing source the main problem in the use of DDGS as a feed component is the high variability of nutrient concentration and quality among different DDGS sources.

Productive performance and egg quality

Mortality was low (< 2.5%) and unrelated to treatment. Egg production, egg weight, egg mass, feed intake, FCR and BW at the end of the trial are shown in Table 3. Hens fed the DDGS-10 and DDGS-15 diets yielded similar (P > 0.05) laying percentage, egg mass, as well as feed intake, FCR and final BW, compared with hens fed 0% DDGS.

Table 3. Egg production parameters and final BW of Arbor Acres broiler breeder hens fed diets with various DDGS levels from 26 to 39 weeks of age

Treatment[1]	Laying percentage, %	Egg weight, g	Egg mass, g/d	Feed intake, g/d	FCR, g/g egg	Final BW, g
DDGS-0	71.23	56.73	40.41	169.57	4.20	3362
DDGS-10	70.80	57.10	40.43	171.34	4.24	3378
DDGS-15	71.96	56.49	40.65	172.08	4.23	3366
SEM[2]	2.18	0.29	0.45	0.96	0.03	16.89
P-value	0.225	0.343	0.177	0.454	0.672	0.534

[1]DDGS-0 = control treatment (corn-wheat-soybean meal basal diet); DDGS-10 and DDGS-15 represents the basal diet (DDGS-0) containing DDGS at 10% (DDGS-10), or 15% (DDGS-15); [2]SEM - standard error of means.

In previous study, Mejia et al. (2012) did not observe differences in hen daily egg production and feed intake of broiler breeder hens (Cobb 500) fed diets with 25% DDGS and 3 different levels of digestible lysine intake: 1000, 800 or 600 mg/hen per day. In our current study all the diets were calculated to be isonitrogenous, isocaloric, and with similar content of TSAA, lysine and tryptophan, calcium and available phosphorus. Krawczyk et al. (2012), also, demonstrated that DDGS (up to 10%) can serve as a useful source of protein in the nutrition of hens included in the genetic resources conservation program because it improves laying performance. On the other hand, Lumpkins et al. (2005) and Roberson et al. (2005), when conducted experiments on laying hens incorporating up to 15% DDGS, reported no negative effect on egg production and feed intake. Results reported by Loar et al. (2010) noted that DDGS does not negatively affect production results of laying hens even at a level of DDGS as high as 32%.

Additionally, Masa'deh et al. (2011) showed that feeding DDGS up to 25%, had no effect on hen egg production and feed intake. On the contrary, Świątkiewicz and Koreleski (2006) reported a reduction in egg production for hens fed 20% DDGS in phase 2 (44-68 weeks of age) of egg production, but not in phase 1 (26-43 weeks of age).

In the present study, corn DDGS-10 or DDGS-15 diets fed to Arbor Acres broiler breeder hens did not adversely affect egg quality characteristics (P > 0.05), except for yolk color (P < 0.001; Table 4), which significantly increase as the dietary level of DDGS increased. According to our results, Masa'deh et al. (2011) also reported that corn DDGS at up to 25% dietary levels, during egg production cycles (Bovan Single Comb White Leghorn-type), had no negative effects on Haugh units, or specific gravity, and improved yolk color at the highest levels. In our experiment, the inclusion of DDGS (up to 15%) in broiler breeder hens' diets increase linearly yolk color. This indicates that dietary DDGS can make the yolk color more intense. Also, ESG (an indicator of eggshell quality) was similar among dietary treatments.

This finding is consistent with the report of Cheon et al. (2008) who found no differences in eggshell weight between layer hens (Hy-line Brown) fed a diet with 0, 10, 15 and 20% of DDGS. Krawczyk et al. (2012) noted that a diet containing 10% DDGS increased yolk color intensity and Haugh units while having no effect on eggshell quality in hens from two different local breeds. This indicates that xanthophylls in the DDGS were highly available. The carotenoid pigments especially xanthophylls contents of corn and DDGS are 17 mg/kg (NRC, 1994) and 30 mg/kg (Roberson et al., 2005), respectively, alleviating the need to use extra pigments in layer diets. The increase in yolk color intensity also, found in the earlier studies by Roberson et al. (2005), Świątkiewicz and Koreleski (2006), Loar et al. (2010), and Masa'deh et al. (2011) who indicates a high availability of carotenoids found in DDGS. Additionally, in an own previous study (Ciurescu et al., 2002) with laying hens (ROSO-SL; peak egg production) fed 30% corn DDGS resulted similar egg production and egg quality, compared with hens fed soybean meal diet. Furthermore, DDGS can contribute as much as half of the protein needed in the laying hen. Our results are partially in agreement with the observations of Lumpkins et al. (2005) who found no significant differences in yolk color, and interior egg quality, as measured by Haugh units, and the eggshell quality, as indicated by the shell breaking strength or specific gravity of the eggs between hens (Hy-line White Leghorn-type) fed diet with 0 or 15% of DDGS.

Reproductive Performance

Reproductive performance was estimated by determining fertility, hatchability of fertile eggs, embryonic mortality and initial growth of the progeny (Table 5).

Fertility and hatchability are the major parameters of reproductive performance which are most sensitive to genetic influences (Liptoi and Hidas, 2006). Fertility refers to the percentage of incubated eggs that are fertile while hatchability is the percentage of fertile eggs that hatch.

The statistical analysis of data of incubated eggs (n = 1,300) showed no significant differences among dietary treatments for hatchability (P > 0.05) but tended to improved fertility percentages (P = 0.085). Fertility numerically improved about 2.40% and 0.87% for the hens fed diet contained 15 and 10% DDGS, respectively than those no fed DDGS. Hatchability of fertile eggs was similar between treatments, possibly because in germ or in shell dead yielded similar results among all treatments (Table 5).

Table 4. Egg quality characteristics of Arbor Acres broiler breeder hens fed diets with various DDGS levels from 26 to 39 weeks of age

Treatment[1]	AH, mm	YW, g	Yolk colour,	AW, %	ESW, %	ESG, unit
DDGS-0	5.83	16.11	7.43 [c]	61.48	10.38	1.088
DDGS-10	5.90	16.07	7.96 [b]	61.46	10.31	1.086
DDGS-15	6.01	16.13	8.28 [a]	61.47	10.16	1.089
SEM[2]	0.66	0.05	0.83	1.07	0.07	0.08
P-value	0.341	0.372	<0.001	0.478	0.265	0.197

Abbreviations: AH, albumen height, YW, yolk weight, AW, albumen weight, ESW, eggshell weight, ESG, egg specific gravity. [1]DDGS-0 = control treatment (corn-wheat-soybean meal basal diet); DDGS-10 and DDGS-15 represents the basal diet (DDGS-0) containing DDGS at 10% (DDGS-10), or 15% (DDGS-15). [2]SEM - standard error of means. [a, b, c] Means within columns with different superscript letters are different (*P* < 0.05).

Table 5. Reproductive performance of Arbor Acres broiler breeder hens fed diets with various DDGS levels from 26 to 39 wk. of age

Treatment[1]	Fertility, %	Hatchability of fertile eggs, %	Dead in germ, %	Dead in shell, %	Chick initial BW, g/chick
DDGS-0	91.7	87.6	3.89	1.91	38.9
DDGS-10	92.5	88.1	3.76	1.89	39.2
DDGS-15	93.9	88.7	3.78	1.87	38.8
SEM[2]	3.22	0.93	0.85	0.42	0.67
P-value	0.085[T]	0.177	0.535	0.282	0.383

[1]DDGS-0 = control treatment (corn-wheat-soybean meal basal diet); DDGS-10 and DDGS-15 represents the basal diet (DDGS-0) containing DDGS at 10% (DDGS-10), or 15% (DDGS-15); [2]SEM - standard error of means.

Our results, also, showed that no significant differences were found among dietary treatments in chicks' post-hatch weight as percent of egg weight (P>0.05).
It is well known that egg weight is positively correlated with chick weight (Wilson, 1991; McNaughton et al., 1978; Tufft and Jensen, 1991).

CONCLUSIONS

On the basis of the results from our current study, inclusion levels of 15% of DDGS in Arbor Acres broiler breeder diets had no adverse effect on egg production and quality characteristics of the egg. Moreover, the use of DDGS improved significantly yolk color intensity and had a positive effect on reproductive parameters results, such as fertility percentage. Therefore, the research demonstrates that corn-DDGS may be an acceptable feed ingredient to be used in broiler breeder diets, replacing a part of imported soybean meal and corn or other cereals. In addition, DDGS might be advantageous to nutritionists in formulating a breeding hen diet at a lower cost.

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POTENTIAL IMPACT OF CLIMATE CHANGE ON AGROECOSYSTEMS IN THE REPUBLIC OF MOLDOVA

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Abstract

This article discusses the assessment of the potential impact of climate change on agroecosystems in the Republic of Moldova. The final results obtained are based on the analysis and comparison of multiannual precipitation and average air temperature (observation period 2016-2019), in the assessment and forecast of the average crop yield. The high thermal regime and the significant deficit of precipitations contributed to the drying of the upper layer of the soil, creating unfavorable conditions for sowing the cereal crops of autumn on most of the territory of the country. The territory of the Republic of Moldova, by its geographical location, is located in the area with insufficient and unstable humidity. The instability of agrometeorological conditions essentially determines the variability and level of crop yield. Agrometeorological investigations were carried out regarding the current situation and the evolution of the meteorological conditions in the territory of the Republic of Moldova in 2019, according to the data of the State Hydrometeorological Service. The average harvest for the last 10 years of sugar beet constituted 310 q/ha; maize for grains constituted 34 q/ha; of sunflower seeds constituted 17 q/ha; of autumn wheat constituted 29 q/ha. The agricultural producers, as well as the public authorities, need a forecast of the productivity of the field crops in the desert in order to take the respective measures. Soil is the key to providing water, energy and building resilience to climate change. Therefore, the international community must take urgent measures to prevent the increasing loss of fertile land. The balance of the humus is profoundly deficient, the reserves of humus decrease annually by about 1t/ha, the nutrients by 180-200 kg/ha. Analysis of the previous weather conditions, forecasts and specialized agrometeorological calculations show that at the beginning of spring the productive moisture reserves in the soil layer with a thickness of 1 m on the fields with autumn crops and autumn plows will constitute 115-160 mm deep (80-115% of the norm).

Key words: *agroecosystem, agriculture, climate change, harvest, Republic of Moldova.*

INTRODUCTION

Currently, among the most valuable ecosystem services directly used by humankind are food, wood and water supply, drought and flood regulation services, as well as rest and tourism. A number of factors influences the variety and vulnerability of biodiversity in the Republic of Moldova (Крупеников, 1989). Due to the geographic location of the country approximately the Carpathian Mountains, the Black Sea and the East-European Plain, a confluence of three main ecoregions is observed here: the Central-European hardwood forests, the Mediterranean forested steppe and the Euro-Asian steppe (Botnari et al., 2011). This confluence of ecoregions provides conditions for high biodiversity. However, ecosystems strongly affected by human use make up about 75% - agricultural ecosystems and 10% urban ecosystems. Natural ecosystems comprise about 15% of the country's surface (Daradur et al., 1996; Nedealcov, 2012). Due to the fact that Climate Change can become a major threat to the functioning of the ecosystem in the long term, at present, ecosystems in the Republic of Moldova are in danger and are degrading due not only to human activity, but also to social-economic conditions, poverty and insufficient political will (Boian, 2015; Урсу, 1990). Practices such as intensive irrigation, the use of chemical fertilizers, pesticides and fungicides, the use of massive agricultural *equipment*, etc. Lead to the degradation, erosion, settling and drying of the organic matter of the soil of the most.

MATERIALS AND METHODS

For the elaboration of the methodology, the evaluation of the potential impact of climate change on agroecosystems in the Republic of Moldova (www.meteo.md) was analyzed initial data on atmospheric deposition, average air temperature and agricultural crops during 15 years (2016-2019). The initial data were collected from the Agrometeorological Bulletins of the State Hydrometeorological Service and from the Statistical Yearbooks of the Republic of Moldova (Todiraş, 2000; Todiraș et al., 2000; www.clima.md; www.old.meteo.md; www.statistica.md), the results being processed by different statistical methods. The forecast of the harvest was made starting from the fact that under the conditions of the chernozem area, which also includes the territory of the Republic of Moldova, the harvest of agricultural crops is largely determined by the water reserves accumulated in the soil upon deprivation from the atmospheric deposits during the cold years.

Two methods of testing the productivity of agricultural crops (Schmandt et al., 1992) were tested according to:

a) atmospheric precipitation during the period September-March (at the level of the republic, rayon, agricultural household).
b) the humidity reserves in the soil at the beginning of the vegetation period and the quantity of productive rainfall from April to June in a multiannual cycle (at field level, sunny).

RESULTS AND DISCUSSIONS

Scientists warn that, unless urgent measures are taken, by 2060 year, global warming is likely to exceed pre-industrial levels by more than 2°C, and this surge could reach as high as 5°C by the end of the century. Such an increase in global temperature will have devastating effects on nature, causing irreversible changes in many ecosystems and, as such, a decline in biodiversity. Rising temperatures and intensifying weather will also generate huge costs for the EU economy and affect the ability of countries to produce food.

Meteorological and agrometeorological characterization on the territory of the Republic of Moldova during the years 2016-2019. *In the Republic of Moldova, the year 2016* was characterized by a high thermal regime and with precipitation, within the norm. The average annual temperature of the air constituted in the territory 9.9 and 11.8°C, exceeding the climatic norm by 1.4-2.1°C and it is reported on average every 10-20 years. According to data from the Meteorological Service of the Republic of Moldova (observation period 122 years) the average annual air temperature was 11.2°C (by 1.7°C higher than the norm), thus it was placed on the 4th place in the number of years with temperatures high annual averages (Figures 1, 2 and 3).

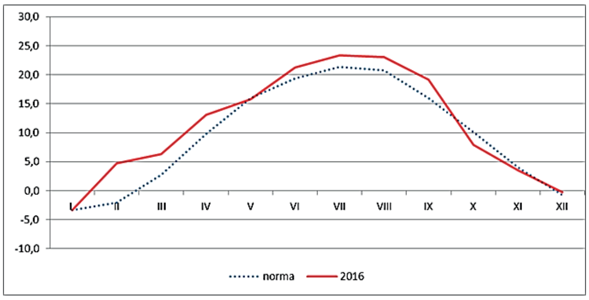

Figure 1. The average monthly air temperature at the Meteorological Service for 2016 (^{0}C)

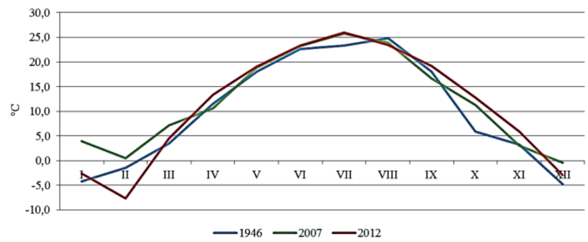

Figure 2. For comparison we have the average monthly temperature of air in 1946, 2007, 2012 (^{0}C)

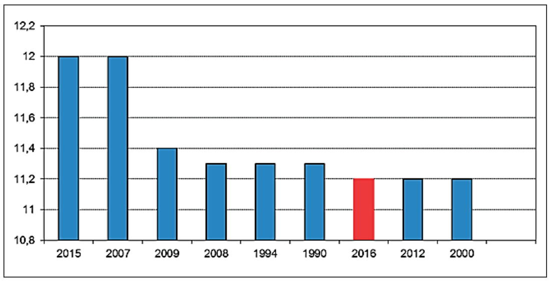

Figure 3. The range of years with high average annual temperatures, according to the Meteorological Service of the Republic of Moldova, 2016 (^{0}C)

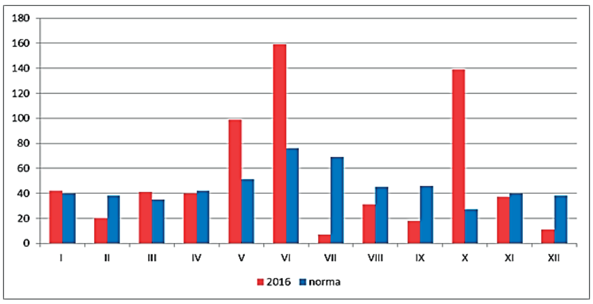

Figure 4. Monthly precipitation at the Meteorological Service of the Republic of Moldova, 2016 (mm)

The monthly precipitation amount per year fell on the territory 445-765 mm or 100-145% of the norm (Figures 4 and 5). The 2015-2016 winter season was short and very hot. The average air temperature for the season was 0.6 and 2.0ºC in the territory, which on a large part of the territory is 3-4ºC higher than the norm and is reported on average every 20-30 years. The amount of precipitation during the season on 60% of the territory of the country constituted 75-117 mm (80-120% of the norm), only at the Camenca Meteorological Service their amount reached 127 mm (150% of the norm). The rest of the territory, predominantly in the central and southern districts, fell 49-70 mm (50-75% of the norm).

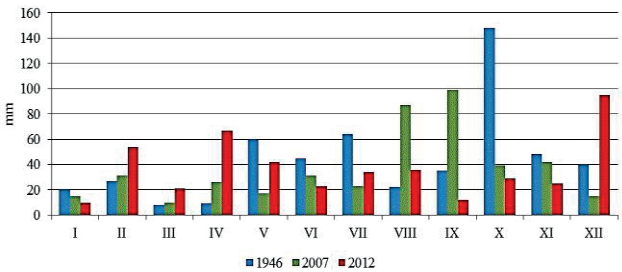

Figure 5. Monthly precipitation quantity in 1946, 2007, 2012 (mm)

In 2016 the average autumn wheat crop in the Republic was 31 q/ha, being higher than the average fruit of the last 10 years by 4 q/ha; maize - 34 q/ha, being 5 q/ha higher than the average fruit of the last 10 years, sunflower - 19 q/ha, being 4 q/ha higher than the average fruit of the last 10 years. The average sugar beet harvest was around 315 q/ha, which is close to the average harvest of the last 10 years. *In the Republic of Moldova, the year 2017* was characterized by a high thermal regime and with precipitation quantities within the norm limits.

The average annual air temperature was 9.8 and 11.5°C (Figure 6), exceeding the climatic norm by 1.2-2.0°C (Figure 7) and is reported on average once in 10-15 years for the multiannual period, and in the last 10 years - on average once in 2 years.

According to the data of the Meteorological Service on the Republic of Moldova (observation period 123 years) the average annual air temperature constituted 11.2°C (by 1.7°C higher than the norm) and was placed on the 4[th] place in the number of years, with high average annual temperatures (Figure 8).

Figure 6. Average annual air temperature at the Meteorological Service of the Republic of Moldova (^{0}C)

Figure 7. The deviation from the norm of the average annual air temperature at the Meteorological Service of the Republic of Moldova (^{0}C)

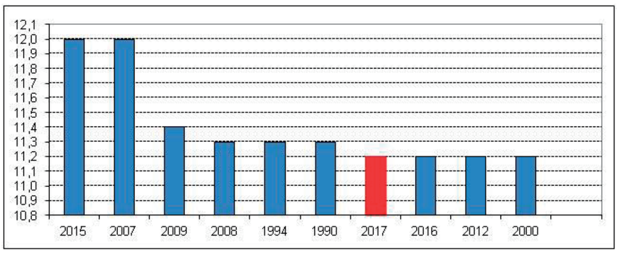

Figure 8. The range of years with high average annual temperatures, according to the Meteorological Service of the Republic of Moldova, 2017 (^{0}C)

The annual amount of precipitation dropped on 85% of the territory constituted 440-650 mm, or 80-120% of the norm (Figures 9 and 10). In the rest of the territory their amount reached 675-700 mm (130-145% of the norm).

The absolute maximum air temperature during the year reached 39ºC (August), which in the summer period is reported on average once in 10 years. The absolute minimum was -21ºC (February), which in winter is recorded on average once in 2-3 years.

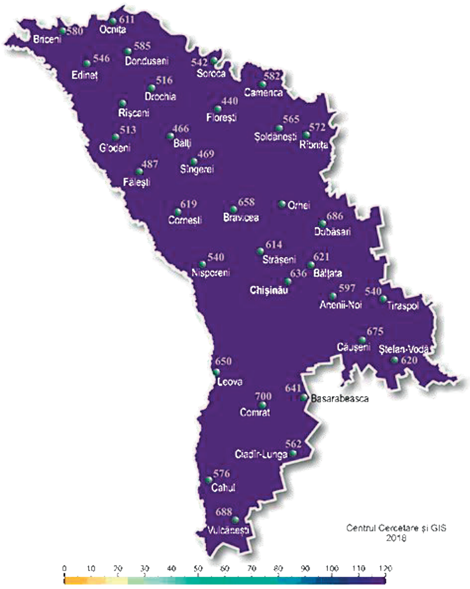

Figure 9. Annual amount of precipitation, according to the Meteorological Service of the Republic of Moldova, 2017 (mm)

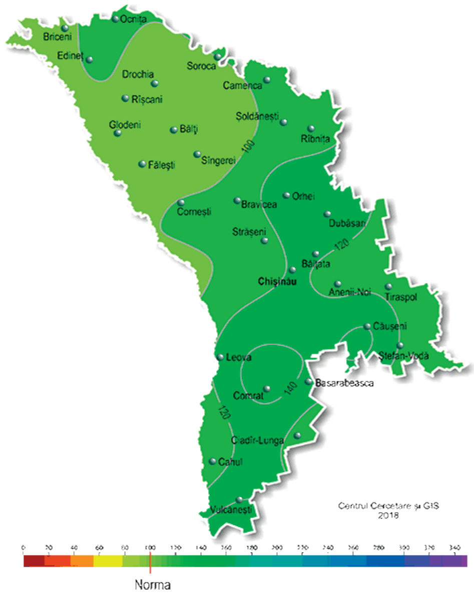

Figure 10. The ratio to the norm of the amount of annual precipitation, according to the Meteorological Service of the Republic of Moldova, 2017 (%)

However, the rainfall during the year fell unevenly. Particularly many precipitations were reported in April (Figure 11), when their monthly amount on 60% of the territory constituted 85-128 mm (230-350% of the monthly norm), which in April is reported for the first time in the entire multiannual measurement period.

In 2017, the average wheat harvest in the autumn constituted in the circus republic 39 q/ha, being higher than the average fruit of the last 10 years by 12 q/ha; maize - 35 q/ha, being 6 q/ha higher than the average fruit of the last 10 years, sunflower - 22 q/ha, being 6 q/ha higher than the average fruit of the last 10 years.

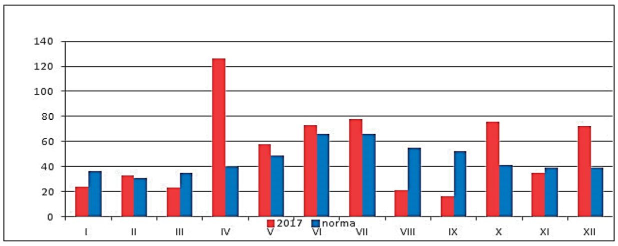

Figure 11. Monthly precipitation, according to the Meteorological Service of the Republic of Moldova, 2017 (mm)

The average sugar beet harvest was around 320 q/ha, being 20 q/ha higher than the average fruit of the last 10 years.

In the Republic of Moldova, the year 2018 was characterized by a high thermal regime and with the annual amount of precipitation within the norm limits. The average annual air temperature in the territory was 9.8 and 11.7°C (Figure 12), exceeding the climatic norm by 1.2-2.1°C (Figure 13) and is reported on average once in 10-15 years for the multiannual period, and in the last decade - on average once in 2 years.

Figure 12. Average annual air temperature at the Meteorological Service of the Republic of Moldova, 2018 (^{0}C)

According to the data of the Meteorological Service (observation period 125 years) the average annual air temperature constituted

11.2°C (by 1.7°C higher than the norm) and was placed on the 4th place in the number of years with average annual temperatures high (Figure 14).

Figure 13. The deviation from the norm of the average annual air temperature at the Meteorological Service of the Republic of Moldova, 2018 (^{0}C)

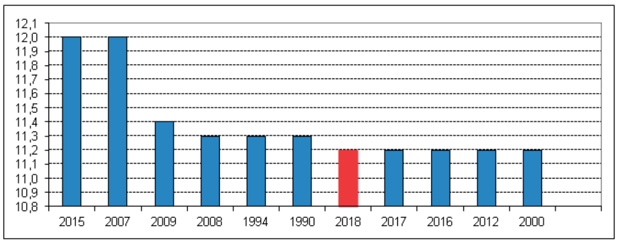

Figure 14. The range of years with high average annual temperatures, according to the Meteorological Service of the Republic of Moldova, 2018 (^{0}C)

The absolute maximum air temperature per year reached 37ºC (August, the Meteorological Service).

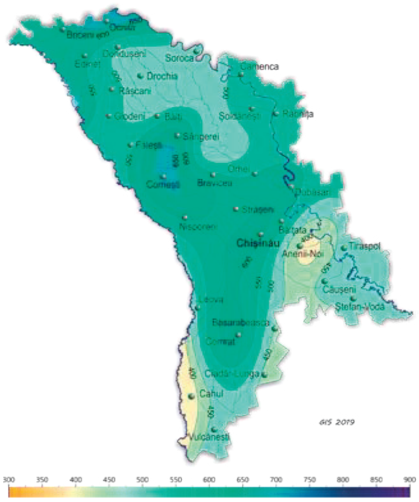

Figure 15. Annual amount of precipitation, according to the Meteorological Service of the Republic of Moldova, 2018 (mm)

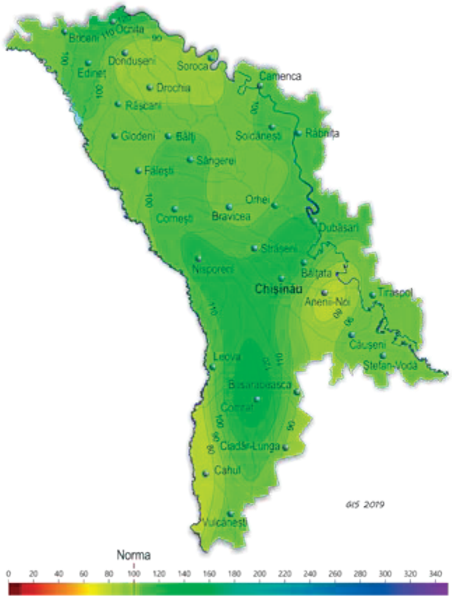

Figure 16. The ratio to the norm of the amount of annual precipitation, according to the Meteorological Service of the Republic of Moldova, 2018 (%)

But the rainfall during the year fell unevenly. Particularly many precipitations compared to the norm were reported in March (Figure 17), when their monthly amount constituted in the bottom 60-120 mm (200-420% of the monthly norm), which on 65% of the territory was reports on average once in 20-30 years.

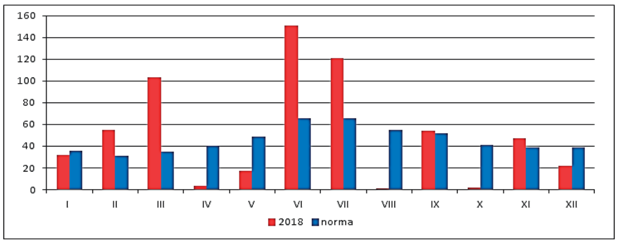

Figure 17. Monthly precipitation, according to the Meteorological Service of the Republic of Moldova, 2018 (mm)

Significant precipitation deficit was reported in April, when their monthly amount on 80% of the territory of the country did not exceed 1-5 mm (5-15% of the monthly norm), isolated precipitations did not fall. The amount of precipitation during the April-May period constituted 10-50 mm in the territory (10-55% of the norm), which is reported on average every 10-30 years. Significant precipitation deficit was also maintained in August and October - their amount in these months did not exceed 1-20 mm (2-40% of the monthly norm). During the vegetation period on 70% of the territory the Republic fell 275-460 mm of precipitation (75-110% of the norm), in the rest of the territory, mainly in the southern districts - 175-255 mm (50-65% of the norm). In 2018, the average harvest of autumn wheat constituted in the circus republic 31 q/ha, being close to the average fruit harvested in the last 10 years and 8 q/ha lower than last year's harvest; maize - 55 q/ha, being 20 q/ha higher than the average fruit of last year and the fruit of the last 10 years, sunflower - 22 q/ha, being 6 q/ha higher than the average fruit from the last 10 years. The average harvest of sugar beet was around 400 q/ha, being 90 q/ha higher than the average fruit of the last 10 years.

The winter season 2018-2019 in the Republic of Moldova was hot and with precipitation (Figures 18 and 20). The beginning of the meteorological winter (stable passage of the average daily air temperature through 0ºC) was reported on the territory on November 17-22, being 10-20 days earlier than usual, and its end - on January 28-29, with a month earlier than average multi-year data. The average air temperature for the season constituted in the territory -1.3 and 0.4ºC, being 1.3-2.0ºC higher than the norm and is reported on average once in 3-5 years.

Figure 18. Average air temperature for the winter season, according to the Meteorological Service of the Republic of Moldova, 2019 (^{0}C)

The absolute minimum of the air temperature constituted on the territory -17ºC (December, Weather Service in Tiraspol, January in Bravicea city). The absolute maximum reached 17ºC (February, Meteorological Service from Balti, Falesti, Corneşti, Tiraspol, Ceadâr-Lunga cities).

Figure 19. The deviation from the norm of the average air temperature for the winter season, according to the Meteorological Service of the Republic of Moldova, 2019 (^{0}C)

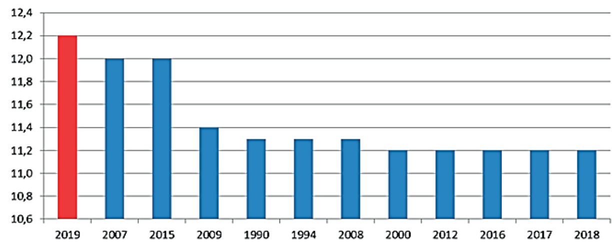

Figure 20. The range of years with high average annual temperatures, according to the Meteorological Service of the Republic of Moldova, 2019 (^{0}C)

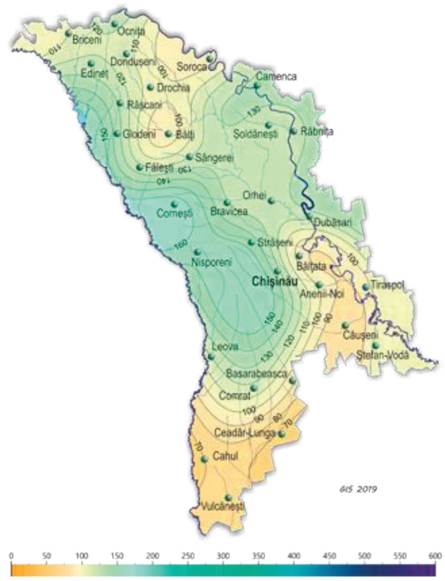

Figure 21. The amount of precipitation for the winter season, according to the Meteorological Service of the Republic of Moldova, 2019 (mm)

The amount of precipitation during the season (Figures 21 and 22) constituted in the territory in the bottom 80-165 mm (80-160% of the norm). Only in some southern districts fell 65-70 mm (70% of the norm).

Particularly many precipitations fell in January, when their monthly amount amounted to 50-85 mm (150-300% of the monthly norm).

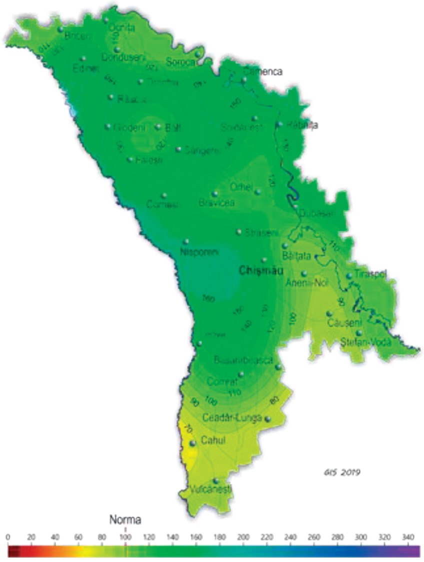

Figure 22. The ratio to the norm of the amount of annual precipitation, according to the Meteorological Service of the Republic of Moldova, 2019 (%)

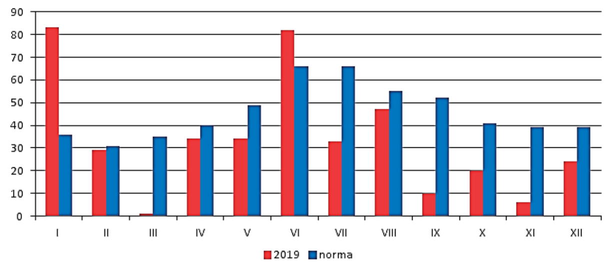

Figure 23. Monthly precipitation, according to the Meteorological Service of the Republic of Moldova, 2019 (mm)

The absolute minimum was on the territory -23ºC (in January in Bravicea city, in February in Soroca city), which during the winter is recorded on average once in 3 years. The annual amount of fallen precipitation constituted in the territory 400-650 mm, or 80-120% of the norm (Figures 15 and 16).

As a result of the agrometeorological investigations carried out regarding the current situation and the evolution of the meteorological conditions for the later phenomena of the sunflower, the State Hydrometeorological Service forecasts for the year 2019 on average for the country a fruit of about 18-20 q/ha (the average fruit for the last 10 years of constituted 17 q/ha); a maize fruit for grains of about 35-40 q/ha (the average fruit for the last 10 years constituted 34 q/ha); a fruit of the sugar beet of about 320-350 q/ha (the average fruit for the last 10 years constituted 310 q/ha); an autumn wheat fruit of about 28-31 q/ha (the average fruit for the last 10 years constituted 29 q/ha).

From a meteorological point of view, the beginning of the winter season of 2020 in the Republic of Moldova is considered the stable passage of the average daily air temperature by 0 ºC in the direction of its decrease, which is reported on average between November 28 (Briceni city) - December 13 (Cahul city). However, depending on the particularities of the synoptic processes, in some years the date of winter establishment can deviate essentially from the average date. There are years when there is no stable passage of air temperature through 0ºC in the direction of its decrease, this phenomenon being observed in the north of the country on average once in 10 years, and in the rest of the territory - on average once in 4 years. The earliest winter for the entire period of instrumental observations over most of the territory was established on November 9, 1988. The average duration of the winter season ranges from 80 days in the south to 100 days in the north.

The average monthly air temperature in the territory of the Republic of Moldova varies between -1ºC in the south of the country and -3ºC in its north. The coldest for the entire observation period was February 1929 - the average monthly air temperature ranged from -12.2ºC to -13.7ºC. The hottest was February of 2002 and 2016 - the average monthly air temperature ranged from 3.5ºC to 5.5ºC.

Average daily air temperature values during the month range from -21 to -26ºC (1954, 2012), 10 and 14ºC (1990, 2016). The absolute minimum of the air temperature in February was -32.1ºC (Balti city, February 20, 1954), and the absolute maximum reached 23.3ºC (Tiraspol, February 26, 1990).

The amount of rainfall in February on the territory of the country is on average 23-40 mm, and the number of days with precipitation ranges from 10 to 14 days. The largest amount of monthly precipitation for the entire period of observations reached in the territory the value of 139 mm (Vulcanesti village, 1969), and the daily one - 70 mm (Goian village, 1999). Precipitation falls mainly in the form of snow. The average decadal thickness of the snow layer in February varies between 3 and 11 cm. The maximum thickness of the snow layer on the meteorological platforms reached 71 cm in some days (February 28, 1973, Briceni city). In February, the following phenomena are possible: fog (on average 2-7 days), deposits of chicory and pole (2-6 days), icicles (about 10 days) and viscous (1-2 days). Of the weather phenomena most frequent are observed abundant snowfall, being possible on average once in 5 years. Strong pole and cap depositions are possible on average every 7 years, and strong wind and blizzard - on average every 10 years. Lowering the air temperature to -25ºC and below can be reported on average once in 10-30 years in the northern half of the country and once in 40-60 years - in the south.

CONCLUSIONS

In mild winter years for winter crops are in danger, which is observed relatively frequently. The most dangerous are the hot winds with average daily temperatures of 5°C and above. During them, the vegetation of autumn and apple orchards is resumed, so their subsequent injury is possible due to the sudden drop in temperature. Such monks are observed almost every year. In the northern districts the number of days with hot winds is not high: in 50-70% of the years - less than 5 days per season and only in 10% of the years they last more than 11-20 days. In the rest of the country, the number of days with hot winds increases up to 5-10 (in 35-50% of years). Warm winds longer than 11-20 days occur in 25-30% of years.

The wintering of autumn sowing largely depends on the weather conditions of the autumn-winter period, the resistance of the sowing to the low temperatures and other unfavorable conditions. The well-rooted and well-rooted plants of autumn, which have undergone a good tempering, are able to withstand decreases in soil temperature to the depth of the twinning knot up to -16 and -18ºC.

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BIODIVERSITY STUDIES ON *Pichia kudriavzevii* FROM ROMANIAN SPONTANEOUS FERMENTED PRODUCTS

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Abstract

Yeasts represent a major group within the microbial communities involved in production of fermented foods. Studies regarding yeast diversity during fermentation, focused on Saccharomyces cerevisiae, but Pichia kudriavzevii also represents an important component of these communities. P. kudriavzevii is widely distributed in nature, is isolated mainly from natural fermentations and is characterised as a potential biocontrol and wastewater treatment agent. The present work deals with biodiversity studies on four P. kudriavzevii strains from Romanian spontaneous fermented products. The strains were identified using conventional tests and molecular analyses of the rDNA coding region. The intraspecific biodiversity was evaluated by PCR-RAPD using different primers. The dynamics of yeast communities from fermented products involved interspecific comparative analysis of RAPD profiles of our strains and their co-fermenters and determination of the similarity index using the Jaccard coefficient (Sij).
This study represents a preliminary step for characterizing the microbial communities from Romanian fermented products and is essential for the improvement of biotechnological processes associated with food industry.

Key words: *Romanian fermented products, Pichia kudriavzevii, RAPD-PCR, intraspecific/interspecific biodiversity.*

INTRODUCTION

The microorganisms represent the most abundant and ubiquitous group of living organisms found on our planet being involved in numerous global relevant processes. Biodiversity is the main feature of this group and since the `70s many scientists presented it as a central research issue (Griebler and Lueders, 2009). Yeasts are the first microorganism domesticated and successfully used for food processing mainly due to their fermentative properties but also due to their rich content of vitamins (Faria-Oliveira et al., 2013). Although *Saccharomyces cerevisiae* is the most known yeast species involved in the production of fermented foods, many other species were characterized as being essential in microbial communities involved in fermentation processes. During last decades, *Pichia kudriavzevii* (formerly known as *Issatchenkia orientalis*), gained an important role biotechnology due to its specific traits that recommend it as a starter culture for obtaining different products. *P. kudriavzevii* is widely distributed in nature being isolated from soil, fruits surface and natural fermentations (Kurtzman, 2011). This species is considered as GRAS (Generally Regarded as Safe) (Bourdichon et al., 2012) and is frequently used for obtaining traditional fermented dairy products such asgariss (camel milk derived product specific to Sudan), mashita (a special butter produced in Egypt) (Ongol and Asano, 2009) and nunu (dairy product similar to yoghurt from Ghana, Nigeria and Burkina Faso) (Johansen et al., 2019). Apart from its involvement in traditional fermentations, members of this species are well known for their use in industry for obtaining biofuels (Mukherjee et al., 2017; Sankh et al., 2013), succinic acid as well for wastewater treatment (Tondee et al., 2008). Bajaj et al. (2013 reported a *P. kudriavzevii* strain able to produce a killer toxin that inhibits growth of different human pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes.* Others, suggested its potential as enzymes producer being able to synthetize phytase, an enzyme very useful for the individuals that have a cereals dominate food pattern and, therefore, are frequently affected by mineral deficiencies (Hellström et al., 2012). Another application is synthesis of curcumin, a polyphenolic compound used as food additive with antioxidative, antitumor and anti-inflammatory activities (Zhang et al., 2013). *P. kudriavzevii* was also characterized as a potential probiotic agent (Chelliah et al., 2016) presenting high resistance to stress conditions associated with the human gastrointestinal tract. The species is also known for its impressive resistance to stress conditions induced by pH extremes, salt high concentrations and heat shock response (Li et al., 2019). Due to its huge biotechnological potential, the aspects regarding the intraspecific biodiversity and the degree of relatedness between *P. kudriavzevii* strains and their co-fermenters, became an important domain of research. Microbial communities of spontaneous fermented food products represent a great source of resources for improving biotechnological processes based on protection of regional specificity.

The present work deals with biodiversity studies on four *P. kudriavzevii* strains isolated from Romanian spontaneous fermented products.

MATERIALS AND METHODS

Yeast strains

SM3, L3S, M6 yeast strains were isolated from spontaneous fermented foods (fermented milk, sour-crèmeand wine wort) from different counties of Romania (Ilfov, Bistrița-Năsăud, Ialomița) and maintained in a Revco LegaciTM Refrigeration System (Copeland, UK) at -70°C, on Yeast Peptone Glucose (YPG) medium (0.5% yeast extract, 1% peptone, 0.2% glucose) supplemented with 20% glycerol. Prior to any experiment, each yeast strain was cultivated on YPGA (YPG medium with 2% agar) slants for 24 hours at 28°C. In this study we used reference strains: *Rhodotorula glutinis* CMGB-G1 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest), *Saccharomyces cerevisiae* CMGB 234, *Hansenula (Ogataea) polymopha* CMGB 233, *Candida parapsilosis* CMGB-DA1, *Hanseniaspora uvarum* CMGB-M1, *Metschinikowia pulcherrima* CMGB-M3 and *P. kudriavzevii* (*I. orientalis*) CMGB 224.

Morpho-physiological analysis

A series of conventional taxonomy tests were used to identify the yeast strains. The morphological aspect of the colonies was observed using yeast cultures grown for 48 hours on YPGA media at 28°C, using a stereomicroscope SZM-1 (Optika Microscopes, Italy), while the and aspect of the cells and the budding type were analyzed with an optical microscope (MICROS, Austria).

Growth tests were performed under osmotic stress conditions induced by high concentrations of glucose (50% and 60%) at 28°C, and at non-permissive temperatures (20°C; 28°C; 37°C; 60°C). In both cases the medium was represented by YPGA and the results were recorder for 3 weeks (Kurtzman, 2011).

For the urease test, we used a special medium containing phenol red as pH indicator and urea (0.1% peptone; 0.1% glucose; 0.5% sodium chloride; 0.2% potassium phosphate monobasic; 0.0012% phenol red; 2% agar; 2% urea added after sterilization). The positive results were indicated by the color changing from yellow to pink. The strain *Rhodotorula glutinis* CMGB-G1 was used as positive control and *Saccharomyces cerevisiae* CMGB-234 as negative control (Corbu et al., 2018).

Phenotypic phylogeny

For a more accurate physiological identification of the tested strains, we performed phenotypic phylogeny analyses using Biolog Microbial ID System according to the manufacturers` specifications.

Genomic DNA isolation

Genomic DNA was isolated and purified according to Csutak et al., 2014, and the concentration of the DNA extracts was determined using a NanoVue Plus spectrophotometer at λ 260 nm.

PCR-RFLP of the ITS1-5.8S rDNA-ITS2 region

The ITS1-5.8S-ITS2 region was amplified according to Csutak et al. (2014) using ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) primers. The amplicons obtained were than digested with *Cfo* I (5'-GCG/C-3'), *Hinf* I (5'-G/ANTC-3'), *Hae* III (5'-GG/CC-3'), and *Msp* I (5'-C/CGG-3') (10 U/µl, Promega) restriction endonucleases and the restriction fragments were observed by agarose gel electrophoresis (1.7% agarose in 0.5X Tris-Borate-EDTA buffer). The size of the amplicons and restriction fragments was determined using Quantity One program (BioRad) after staining with ethidium bromide in a final concentration of 0.5 µg/mL.

PCR-RAPD

The PCR-RAPD analysis of the newly isolated strains was performed in total volume of 50 µL using 5 primers: OPA03 (5'-AGTCAGCCAC-3'); OPA11 (5'-CAATCGCCGT-3'); OPA18 (5'-AGGTGACCGT-3'); OPE18 (5'-GGACTGCAGA-3'); M13 (5'-AGGGTGGCGGTTCT-3'). The reaction mix comprised 100 ng genomic DNA; 2 mM magnesium chloride ($MgCl_2$), 0.2 mM dNTP mix, 2 µM primer, 1.25 U GoTaq DNA polymerase and 1X Green GoTaq Flexi Buffer (Promega). The PCR program included: initial denaturation 2 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 36°C and 2 min 72°C and a final extension of 5 min at 72°C (Biometra T Gradient Thermocycler). Negative control reactions (without genomic DNA) were also run.

Numerical analysis of the RAPD patterns

The genetic relatedness of the isolates (intraspecific biodiversity) was determined analyzing the RAPD profile obtained for each strain and by calculating the similarity index using the Jaccard coefficient (Sij). Similarly, the interspecific biodiversity of the microbial communities from spontaneous fermented products was determined by comparing their profile to the RAPD profile of their co-fermenters.

Appropriate dendograms were generated by PyElph (Pavel and Vasile, 2012), using UPGAMA (Unweighted Pair Group Method with Arithmetic Mean) method.

RESULTS AND DISCUSSIONS

Yeast strains isolation and morpho-physiological characterization

Spontaneous fermented foods represent an ecological niche characterized by impressive microbial biodiversity. Three yeast strains isolated from different spontaneous fermented foods and beverages from Romania Y-L3S, Y-SM3 and Y-M6 (Table 1) were identified using conventional taxonomy tests. After 48 hours of growth on YPGA medium our strains formed white colonies, elevated and convex with slightly different surfaces. Y-M6 colonies had dried surface (Figure 1, C1), while Y-L3S (Figure 1, A1) and Y-SM3 (Figure1, B1) colonies were smooth. Our strains presented ovoid cells with polar budding (Figure 1 A2; B2; C2).

Table 1.The three newly isolated yeast strains

Yeast strains	Source of isolation	County/Region
Y-L3S	Fermented milk	Ilfov
Y-SM3	Sour-crème	Bistrita-Nasaud
Y-M6	Wine wort	Ilfov

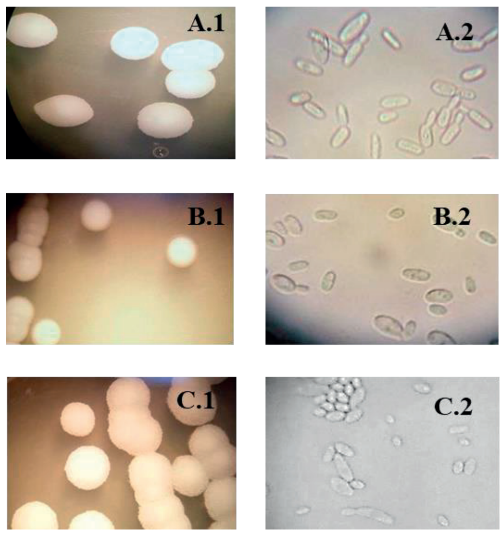

Figure 1. Aspect of the colonies and cells (40X) formed by isolated yeast strains (A.1/A.2- Y-L3S; B.1/B.2- Y-SM3; C.1/C.2- Y-M6)

Physiological characterization of yeast strains is considered a useful tool for taxonomical identification. Also, their behavior in different culture conditions can predict their biotechnological potential. According to Table 2, our strains presented high resistance to stress conditions imposed by temperature variation and high concentrations of glucose. The results are consistent with the description of *P. kudriavzevii* CBS 2911, although this strain showed a reduced growth at temperatures higher than 37°C and slow/delayed growth in presence of 50% glucose. *P. kudriavzevii* is well known for its high resistance to stress conditions, which is related to the pressure of the environment that influences the rate of expression of genes involved in specific metabolic pathways in strains isolated from natural habitats (Wada et al., 2019; Matsushika et al., 2016). Urea hydrolysis is considered an important test that shows dichotomy between different species of yeasts. None of our strains was able to produce urease, the results being correlated with the data from the scientific literature (Kurtzman, 2011) (Figure 2).

Figure 2. Urea hydrolysis test results
A - negative control - *S. cerevisiae* - CMGB 234;
B - CMGB Y-L3S; C - CMGB Y-SM3; D - positive control - *R. glutinis* - CMGB G1; E - CMGB Y-M6

Phenotypic phylogeny

In order to narrow down the number of species possibly corresponding to our three newly isolated strains, we used Biolog Microbial ID System.

The system uses over 90 assimilation and fermentation tests to establish a metabolic profile for the tested strain. When the analysis is over the result is compared to a database with over 250 yeast species and generates phenotypic phylogeny (DeNittis et al., 2010). All three strains were classified in the *P. kudriavzevii* species with a percentage of similarity greater than 90% (Figure 3).

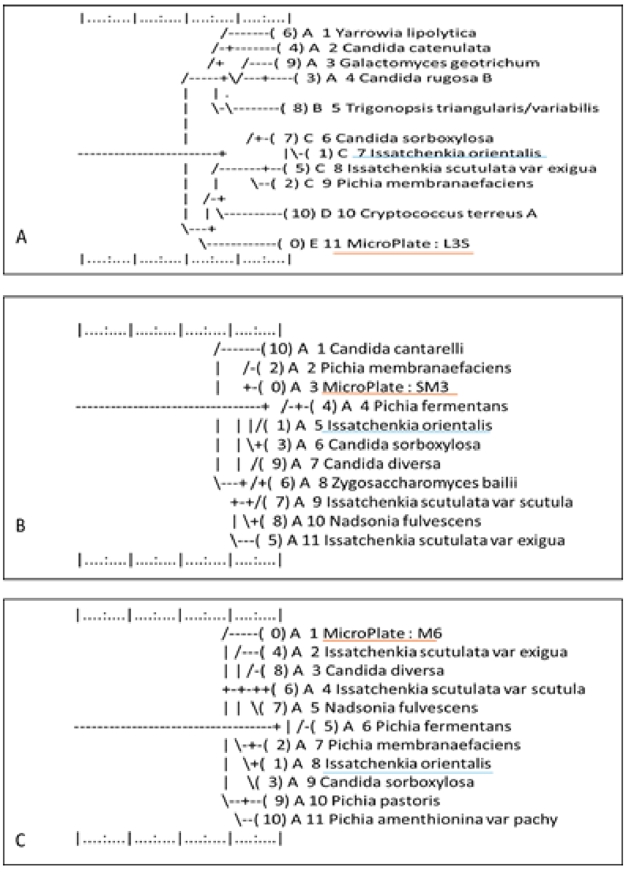

Figure 3. Phenotypic phylogeny generated by Biolog Microbial ID System
(A: Y-L3S; B: Y-SM3; C: Y-M6)

Table 2. Conventional taxonomy tests results

Strain/Species	Urease	Temperatures				Osmotic stress growth	
		20°C	28°C	37°C	42°C	50% glucose	60% glucose
CMGB-Y-L3S	-	+	+	+	+	+	+
CMGB-Y-SM3	-	+	+	+	+	+	+
CMGB-Y-M6	-	+	+	+	+	+	+
***Pichia kudriavzevii* CBS 2911 (Kurtzman et al., 2011)**	-	+	+	+	-	**-/D**	-

Legend: (+) significant growth recorded during first 48 hours of incubation; (-) no growth recorded after 48 hours of incubation; (D) growth was recorded after more than 72 hours of incubation.

Molecular identification

In order to confirm the results obtained by conventional taxonomy techniques we used ARDRA technique to analyze the ITS1-5.8S-ITS2 region.

The strain *P. kudriavzevii* (*I. orientalis*) CMGB 224 (Ghindea et al., 2009) isolated from yogurt, was used as reference for molecular analyses. According to Figure 4, all three strains showed similar restriction patterns for the four used endonucleases, with a few minor differences. The *Cfo* I enzyme generated three fragments of 220, 200 and 75 bp for Y-SM3 and Y-M6 strains and two fragments of 220 and 75 bp for Y-L3S strain. The *Hae* III digestion generated two fragments of 410 and 100 bp, while the *Hinf* I restriction resulted in two fragments of 220 and 160 bp. The *Msp* I enzyme restriction generated only one fragment with approximately 265 bp. The comparison of the restriction patterns obtained for our strains with those from similar studies (Table 3), confirmed the belonging of our strains to *P. kudriavzevii* species.

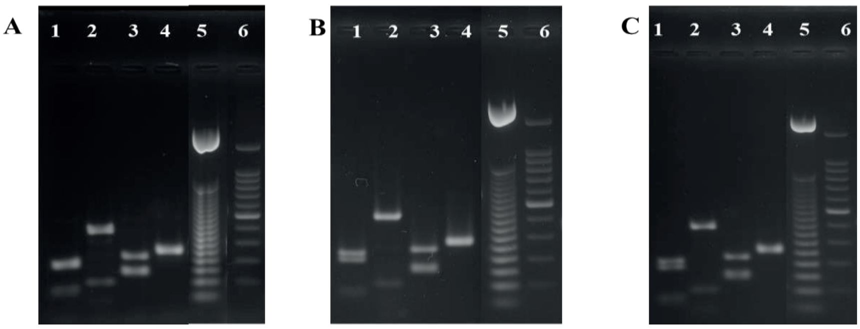

Figure 4. PCR-RFLP of the ITS1-5.8S-ITS2 region for Y-L3S-(A); Y-SM3-(B); Y-M6-(C) strains
Legend: 1-*Cfo* I; 2-*Hae* III; 3-*Hinf* I; 4-*Msp* I;
5-100-bp DNA Ladder (ThermoFisher Scientific); 6-BenchTop-50-bp-DNA Ladder (Promega)

Table 3. Amplicons and restriction fragments of ITS-5.8S rDNA-ITS2 region from isolated and reference yeast strains

Strain/species	**Amplicon (bp)**	***Cfo* I (bp)**	***Hae* III (bp)**	***Hinf* I (bp)**	***Msp* I (bp)**
Y-L3S	520	220; 75	410; 100	220; 160	265
Y-SM3	520	220; 200; 75	410; 100	220; 160	265
Y-M6	520	220; 200; 75	410; 100	220; 160	265
I. orientalis CMGB 224 (Csutak et al., 2012)	520	204; 178; 69; 52; 6	386; 38	218; 154; 137	260; 249
I. orientalis CBS 5147 (Csutak et al., 2012)	509	210; 170; 80	400; 100	210; 150; 150	260; 260
I. orientalis (Nisiotou et al., 2007)	500	200; 190; 70; 50	400; 90	220; 140; 140	ND
I. orientalis (Granchi et al., 1999)	500	185; 170; 69; 56	370; 90	225; 160; 145	ND
P. kudriavzevii VTT C-89178 (Pham et al., 2011)	500	200; 180; 70; 50	360; 90; 50	220; 150; 130	ND
I. orientalis MC10(1) (Basilo et al., 2011)	520	ND	ND	ND	280; 240

Biodiversity of *P. kudriavzevii* strains in fermented products

The RAPD technique is a commonly used molecular method for interspecific and intraspecific studies among different yeast species. RAPD fingerprints are obtained by PCR amplification using very short primers (no longer than 10-mer) and low annealing temperature (Samuelsson et al., 2010). This technique has many advantages such as: no need for prior knowledge of the genome investigated; rather low economic costs; a large range of primers yielding numerous amplicons with high polymorphism which allow determination of intraspecific biodiversity. The main disadvantages of the technique reside in the low possibility of distinguished between homozygotes and heterozygotes; the reproducibility is also problematic due to the fact that the results are strongly influenced by the amplification parameters (*e.g.* DNA concentration/quality and degree of RNA contamination) (Kumari and Thakur, 2014; Csutak et al., 2011).

In this study we used five primers (OPA03; OPA11; OPE18; M13) selected during previous work (data not shown) based on the numbers of bands generated. To overcome the low reproducibility of this technique, we optimized the parameters and the reaction mix using *I. orientalis* (*P. kudriavzevii)* CMGB 224 as reference strain and comparing the results with previous studies (Ghindea et al., 2009). After optimization and confirmation of the reproducibility of the technique, the RAPD-PCR parameters were used to determine the genetic relatedness of the isolates (intraspecific biodiversity) (Figure 5). Similarity was estimated mainly by the presence of a band and not by its intensity (Atienzar and Jha, 2006),

therefore the similarity index based on Jaccard coefficient was preferred in order to estimate the intraspecific polymorphism among members of *P. kudriavzevii* species isolated from different sources. Also, the absence of a specific band generated by a RAPD primer does not imply genetic similarity between two genotypes, the Jaccard`s index being therefore more efficient for RAPD based intraspecific polymorphism analyses (Rabie et al., 2010).
According to Figure 5 all selected primers generated more than four amplification fragments for each strain analyzed except for *C. parapsilosis* CMGB-DA1, *H. uvarum* CMGB-M1 and *M. pulcherrima* CMGB-M3 amplified with OPE18 primer in which case we obtained two respectively a single fragment for the last two strains. The size of the fragments varied from 100 bp to over 2000 bp (determined by PyElph software) (Table 4). Although optimized, the RAPD amplification generated numerous low intensity amplicons. In our analyses these amplicons were omitted. All primers tested yield different degrees of polymorphism between our strains, but the primers OPE18 and M13 seemed to be the most discriminating. Therefore, we considered that these primers could be used for further testing of *P. kudriavzevii* intraspecific biodiversity using a greater number of strains isolated from similar environments but from different regions, in order to determine a possible correlation between these two parameters.

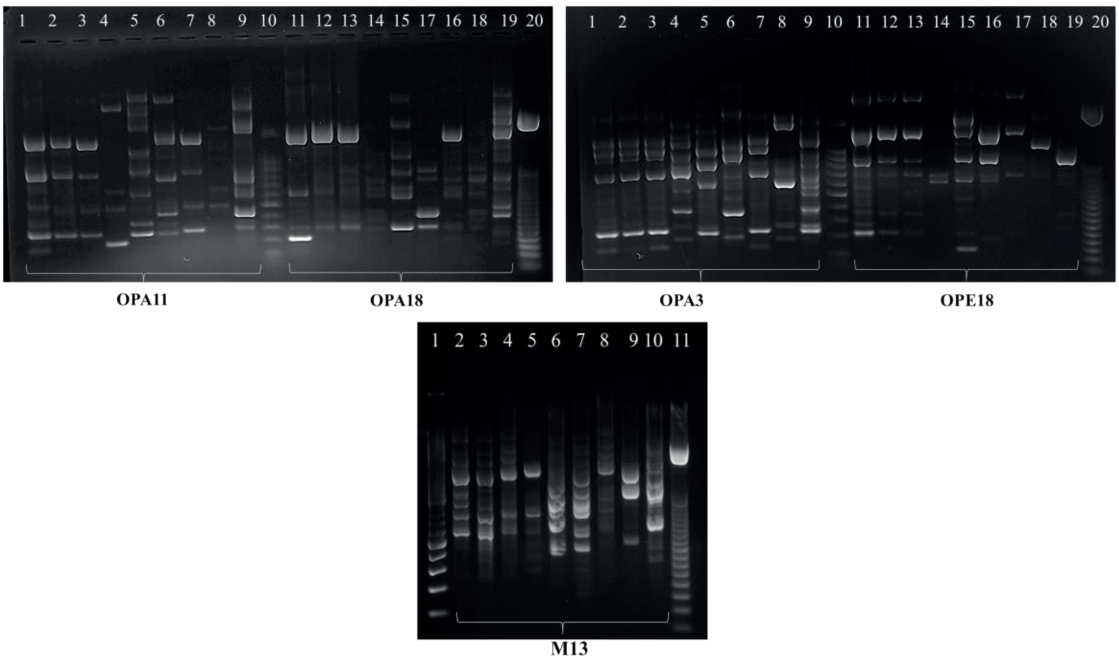

Figure 5. RAPD profile of *P. kudriavzevii* strains and their co-fermenters
Legend: 1/11 - *P. kudriavzevii* CMGB 224; 2/12 - *P. kudriavzevii* Y-SM3; 3/13 - *P. kudriavzevii* Y-L3S; 4/14 - *C. parapsilosis* CMGB-DA1; 5/15 - *H.* (*O.*) *polymorpha*; 6/16 - *S. cerevisiae* CMGB-234; 7/17 - *P. kudriavzevii* Y-M6; 8/18 - *H. uvarum* CMGB-M1; 9/19 - *M. pulcherrima* CMGB-M3; 10 - 100-bp DNA Ladder (ThermoFisher Scientific); 20 - BenchTop-50-bp-DNA Ladder (Promega)

Table 4. Intraspecific polymorphism of *P. kudriavzevii* isolates

Primer	Total bands	Polymorphic bands	Common bands	Jaccard Index
OPA11	19	3	16	84.2%
OPA18	16	2	14	87.5%
OPA03	20	3	17	85%
OPE18	25	6	19	76%
M13	34	9	25	73.5%

In order to obtain a better phylogenetic position of our strains in relation to other fermentative yeast species, the RAPD profiles generated from other strains isolated from similar environments (Figure 5) were used to construct UPGAMA dendrograms (Figure 6). This technique is frequently used in bioinformatics for the creation of phonograms using an agglomerative clustering method. Since we do not have prior knowledge regarding the distance between the pairs of taxa analyzed we decided that a rooted phylogenetic tree is much more suitable for our work. This algorithm examines the similarity matrix and organizes data into clusters without offering information about the evolutionary descendant (Kaur et al., 2013). With the exception of the OPA03 primer, which placed the *P. kudriavzevii* CMGB-M6, isolated from wine wort, in the same cluster with *P. kudriavzevii* CMGB-L3S, *P. kudriavzevii* CMGB-SM3 isolated from dairy products (Figure 6) all the primers indicated significant differences between this strain and the other *P. kudriavzevii* analyzed strains. Although, there are no significant differences between *P. kudriavzevii* CMGB-M6 and *P. kudriavzevii* Y-SM3 and Y-L3S, all three are clearly separated from *P. kudriavzevii* (*I. orientalis)* CMGB 224 reference strain. Therefore, we can consider that the primer OPA03 can be recommended as molecular marker for interspecific biodiversity in terms of fermented food microbiota.

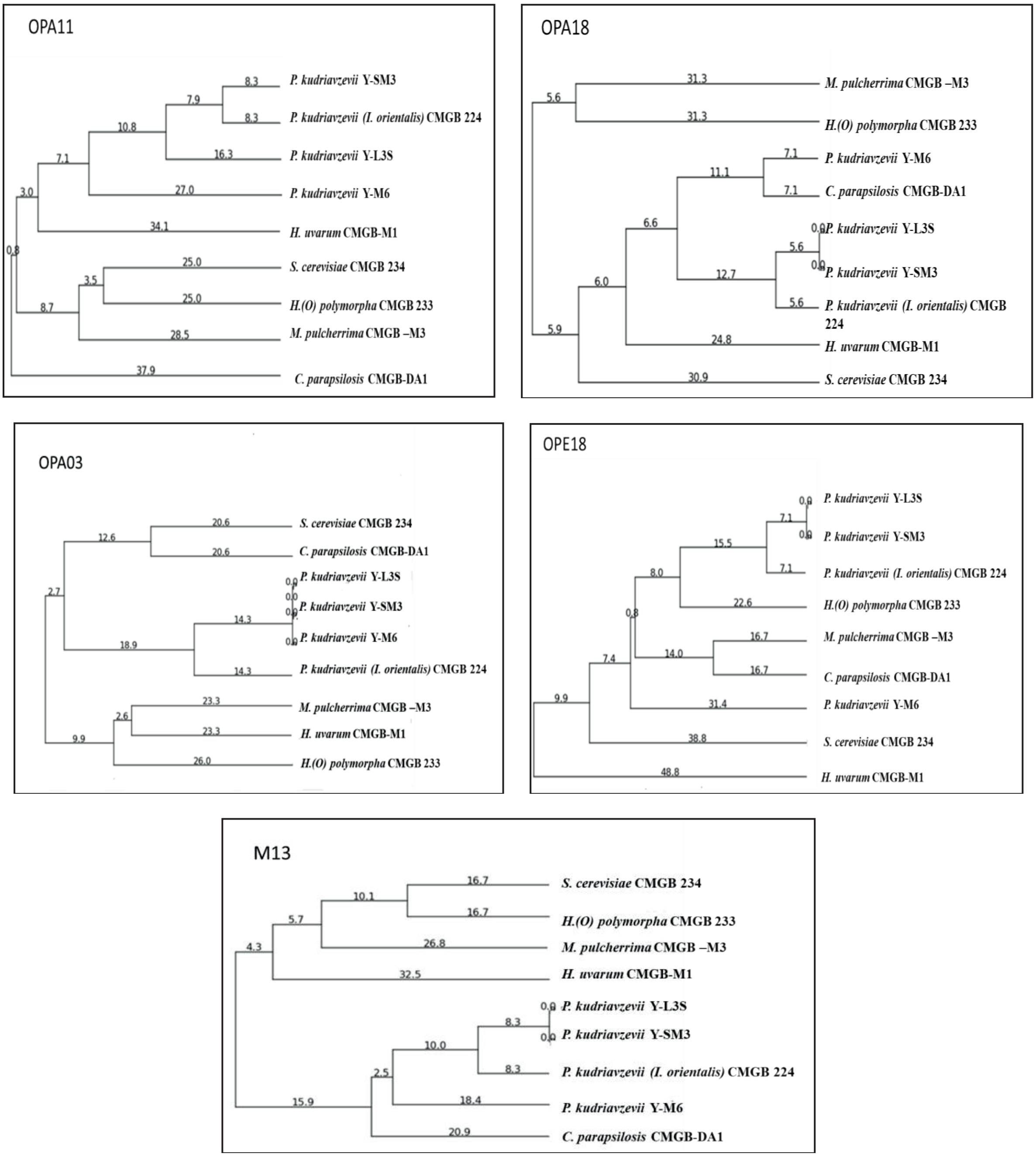

Figure 6. UPGAMA dendrogram for *P. kudriavzevii* strains and their co-fermenters, obtained from RAPD profiles with OPA11, OPA18, OPA03 and OPE18 primers

On the other hand, the primer OPA11 was the only one able to generate profiles allowing the separation of *P. kudriavzevii* Y-SM3 from Y-L3S. Therefore, we consider that a combined analysis of RAPD profiles obtained with OPA03 and OPA11 primers could provide a more accurate evaluation of intraspecific and intergeneric polymorphism.

Both OPA18 and M13 primers placed the strain *C. parapsilosis*CMGB-DA1 strain close to the clusters formed by *P. kudriavzevii* isolates (Figure 6). Moreover, the OPA18 primer placed *P. kudriavzevii* Y-M6 and *C. parapsilosis* CMGB-DA1 on a distinct branch of the same cluster. Until recently, *P. kudriavzevii*was considered the teleomorph form of *C. krusei*. Douglass et al 2018 proved that the two species are synonyms and not distinct, fact that might explain our results (Schmalreck et al., 2014).

Although OPE18 and M13 primers generates the lowest Jaccard`s index (Table 4), according to the dendrograms, these primers are not able to reunite all the *P. kudriavzevii* strains under in the same cluster or to separate them from other species. As a consequence, we believe that these primers are more suitable for analyzing *P. kudriavzevii* intraspecific biodiversity and not for interspecific or intergeneric studies.

CONCLUSIONS

Sustainable development of biotechnological processes should be based on the diversity of microorganisms used and the metabolites they produce. Although, in present there are numerous modern technologies that allow researchers to manipulate and modify microorganisms in order to enhance their industrial performance, sometimes these technologies are regarded with skepticism. Studies on yeasts from natural environments might reveal the existence of different secondary metabolites that could present an economic advantage compared to their synthetic or recombinant version (Singh, 2010). Exploring the biotechnological potential of microorganisms based on biodiversity and ecological impact studies depends on the existence of a modern infrastructure. Technological developed countries which have the possibility to obtain easily patent and to commercialize biological products can benefit from the full potential of microbial diversity (Tripathi et al., 2007). Our study deals with this problem, representing a preliminary step for the optimization of biotechnological processes with industrial value.

The present work deals with the identification and biodiversity of four *P. kudriavzevii* strains isolated from Romanian spontaneous fermented products. The primer OPE18 generated the highest intraspecific varibility among *P. kudriavzevii* isolates, while the OPA3 primer was able to reunite all *P. kudriavzevii* strains in the same cluster. The combination of RAPD analyses using the primers OPA11 and OPA3 is recommended as a relible method for intra- and intergeneric biodiversity studies on yeasts traditional spontaneous food microbiota.

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RESEARCH TRENDS AND GAPS IDENTIFICATION ON FARM MODERNISATION SUPPORT MEASURES IN THE EUROPEAN UNION

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Abstract

Modernization of agricultural holdings is one of the most important measures in the Rural Development Programs, directly addressing the competitiveness of the agricultural sector. The specific funds for modernization accessed through Submeasure 4.1. - Investment in Agricultural Holdings and Measure 121 - Modernization of agricultural holdings are purposed to adapt agricultural holdings to market requirements, with a strong positive impact on agricultural production, on the marketing of agricultural products and also for environmental protection. This paper is intended to investigate the current state of the research conducted hitherto at the EU level and highlight trends in how academic literature has received CAP attempts in addressing the modernization of agricultural holdings. The scientific database Web of Science (WoS) was interrogated using a list of research terms and offering insights into research topics and trend evaluation from different perspectives. The provided data was processed through the CiteSpace software, by generating a series of interactive graphics. Subsequently, the conclusions obtained are used to identify possible future research as well as to conclude a further course of the modernization measures in the EU farms in the next reform of the Common Agricultural Policy.

***Key words**: agricultural holdings, EU funds, RDP, farm modernisation, CiteSpace.*

INTRODUCTION

Fifty-two percent of the European Union's territory is categorized as predominantly rural, having more than 170 million hectares of agricultural land, and 113 million people (nearly one-quarter of the EU population) living in rural areas. An EU rural development policy was set up to meeting the challenges related to the economy, environment and the society faced by the seareas (Augère-Granier, 2016).
Agenda 2000 established a rural development policy known as the second pillar of the Common Agricultural Policy (the "CAP") and brought rural development under a single regulation to apply across the whole of the European Union (European Commission, 2013).
Pillar II, funded by the European Agricultural Fund for Rural Development (EAFRD), is being used by the Member States to achieve a variety of EU rural development policy goals such as: improving the competitiveness of farm, forest and agri-food businesses; helping protect the natural environment; supporting rural economies and quality of life in rural areas through the rural development program measures. Measures under the second pillar are voluntary and co-financed from the national level (Rumanosvska, 2016).
According to the European Parliament, "Pillar II policies have successfully supported inter-generational transfer through retirement and succession schemes and promoted farm modernisation through investment schemes" (Hennessy, 2014).
Modernisation (be it of individual farms or the agricultural sector as a whole) is conventionally thought of as a unilinear trajectory involving scale-enlargement and specialisation in order to achieve productivity increases (Potočnik, 2015).
We can say that the modernisation of agriculture is a process of converting agriculture from conventional labour-based agriculture to technology-based agriculture (Wu, 2011). Also, the topic of modernisation is one of the

basic issues in agricultural policies, especially in countries, where agriculture presents a low level of development (Kusz, 2014).

The farmers receive grant assistance throughout Farm Improvement Schemes (FIS) (Measure 121) for various purposes like investment in buildings and machinery which improves environmental protection, animal welfare, output quality and working conditions. All of these to increase the capacity, competitiveness and innovation of farming through investment in farm modernisation (Hennessy, 2014).

MATERIALS AND METHODS

Taking into account the publications in the field and all the literature regarding the subject of farm modernisation measures in the last two Multiannual Financial Framework, respectively 2007-2013 and 2014-2020, we want to realize a literature review article.

Data was obtained from the ISI Web of Science (WoS) database of Clarivate Analytics, where we searched for articles published between 2007 and 2020.

Our search is made through a key words combination specific for the WoS platform (farm modernisation OR agricultural holdings modernisation OR farm investment AND CAP measures AND Common Agricultural Policy). In this search, OR it means that at least one term must occur to be retrieved while AND it means that all search terms must occur to be retrieved.

The search for those combined key words generated 772 references using the databases Web of Science Core Collection.

Selection criteria

We have narrowed the web of science categories by taking into consideration only eight categories out of one hundred (i.e. Economics, Environmental studies, Agricultural economics policy, Agriculture multidisciplinary, Environmental sciences, Agronomy, Agricultural engineering and Management). Thus with the above-mentioned filters, the research has 298 articles as an outcome.

These results were analyzed using CiteSpace software. CiteSpace allows us to mapping authors, journals (sources) and countries. This software also is helping us to identify and visualize the main factors contributing to the knowledge evolution inthe search field under study (Chen, 2014).

The results of the program are shown as infographic forms, with a node indicating research items, and links between nodes describing citations or mutual references between these nodes. Each node is represented by a series of tree rings in different colors; a spectrum of colors serves to indicate the chronological order of occurrence of links and items (Xiang, 2017).

The paper disregards the scientific articles from the last century because the subject of rural development has been an official objective since the beginning of the 2000s, with the establishment of Pillar II, and the purpose of the paper is to analyze the research on certain measures of this pillar. Should we deem necessary to investigate more a certain article which falls outside the previously set periods and which is in the interest and the necessity to understand the farm modernization, we will be open to further study that specific period.

RESULTS AND DISCUSSIONS

Regarding the interest in this subject, a constant increase was observed in the number of yearly publications, from 16 in 2007 to 44 in 2018, when we have the highest number in Figure1.

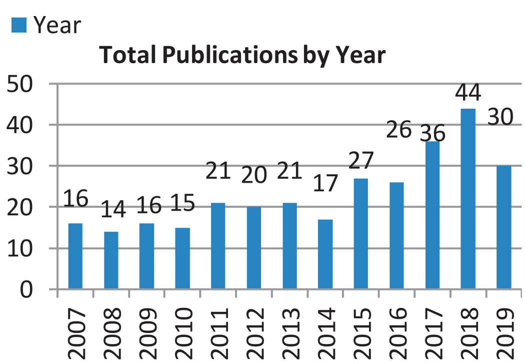

Figure 1. Total Publications by Year
(Source: Web of Science database)

These publications were classified into 5 types of documents: article, proceedings paper, review, book chapter and early access. Articles were predominant comprising 68% of the total. The predominant language is English (92.4%), seconded by Spanish (2.3%), English being the preferred language of communication in the scientific community.

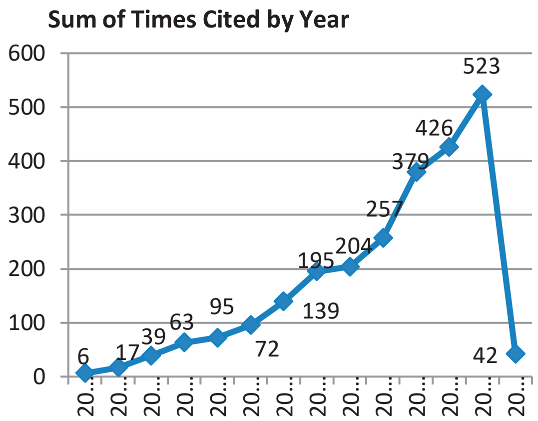

Figure 2. Sum of Times Cited by Year
(Source: Web of Science database)

Our result articles in from dataset were cited 2,457 times, with an average of 8.11 citations per item. The situations in which the articles were cited are increased constantly year by year (Figure 2).

The value of H-index is 24, suggesting that this line of research has a relevant impact factor. The H-index was developed by Hirsch in 2005 and reveals the impact or relevance of a paper, author or field (Hirsch, 2005).

In Figure 3, information on the number of citations and cooperation networks between countries is summarized, showing the U.S. as the top-ranked country. This figure can also expose a lack in terms of cooperation between countries. A more solid connection between two or more countries it means the cooperation between those countries is at a higher level (Zuanazzi, 2019). The clusters are presented in different colors, as we can see in Figure 3.

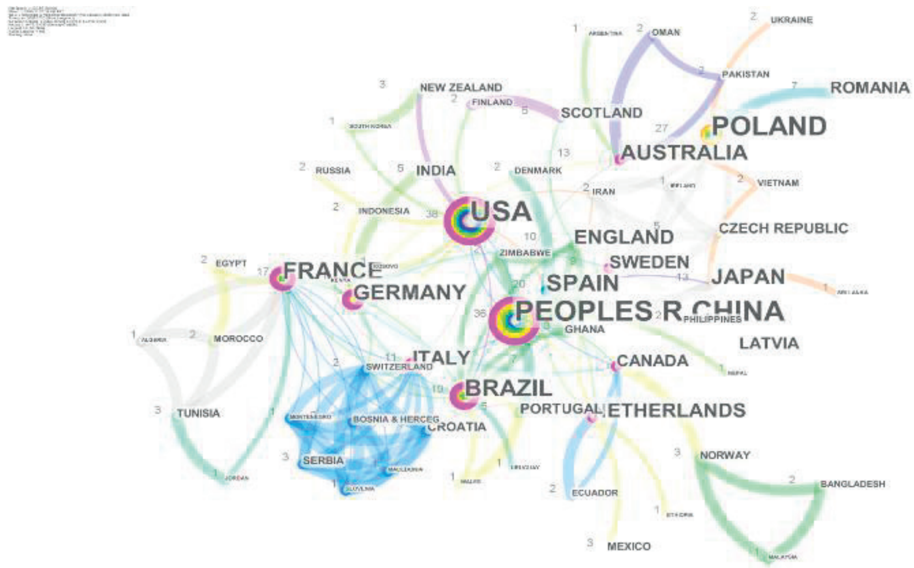

Figure 3. Geolocalization of scientific papers about farm modernisation, agricultural holdings modernisation, farm investment. Different colors mean different clusters and the numbers represent the citation counts
(Source: CiteSpace platform)

The numbers represent the citation count of each country. The top-ranked article by citation counts is the U.S., with a citation counts of 38, the second is China (36), and the third is Poland (27).

Table 1 presents the centrality of some countries. Centrality is an important measure that varies from 0 to 1, repres
enting the influence of a country (Chen, 2015). In this case, the USA has the greatest centrality (0.27), followed by the Republic of China (0.21) and Germany (0.26).

Table 1. The centrality of the countries

Count	Centrality	Year	Countries
38	0.27	2007	USA
36	0.21	2007	PEOPLES R CHINA
27	0.08	2007	POLAND
20	0.09	2010	SPAIN
19	0.20	2008	BRAZIL
17	0.23	2009	FRANCE
13	0.14	2010	AUSTRALIA
13	0.26	2010	GERMANY
13	0.06	2007	JAPAN
12	0.13	2007	NETHERLANDS

Source: CiteSpace platform

Besides this, some countries presented citation bursts. A citation burst is an indicator of a most active region (in this case), which can last for multiple years as well as a single year.
This indicator provides evidence that a publication/country is associated with a wave of citations. In other words, the item has evidently attracted an extraordinary point of attention from its scientific community (Chen, 2015).
From this point of view, the top-ranked item is the USA (2008-2011) with bursts of 4.67.
The second place is occupied by France (2014-2016) 3.09 and on the third place, there is the Republic of China (2014) with a strength of 2.08, as we can see on Table 2.
Regarding the categories selected from Web of Science, based on the analysis of occurrence, we can see which area is highlighted in our research. The information has been analysed using the CiteSpace platform.

In Figure 4 the node represents a subject category (where the purple rings represent centrality and the different colored lines mean different clusters), while a line connecting the two nodes demonstrates the co-occurrence of two subject categories.

Table 2. Countries with citation bursts

Top 23 Countries with the Strongest Citation Bursts

Countries	Year	Strength	Begin	End	2007 - 2020
USA	2007	4.6735	**2008**	2011	
INDIA	2007	1.2873	**2011**	2011	
PAKISTAN	2007	1.2873	**2012**	2012	
CANADA	2007	1.8251	**2013**	2013	
GREECE	2007	1.2972	**2014**	2014	
FRANCE	2007	3.0913	**2014**	2016	
PEOPLES R CHINA	2007	2.0826	**2014**	2014	
AUSTRALIA	2007	1.4501	**2015**	2017	
LITHUANIA	2007	1.2378	**2015**	2015	
ROMANIA	2007	1.87	**2015**	2015	
LATVIA	2007	1.125	**2016**	2016	
ENGLAND	2007	1.125	**2016**	2016	

Source: CiteSpaceplatform

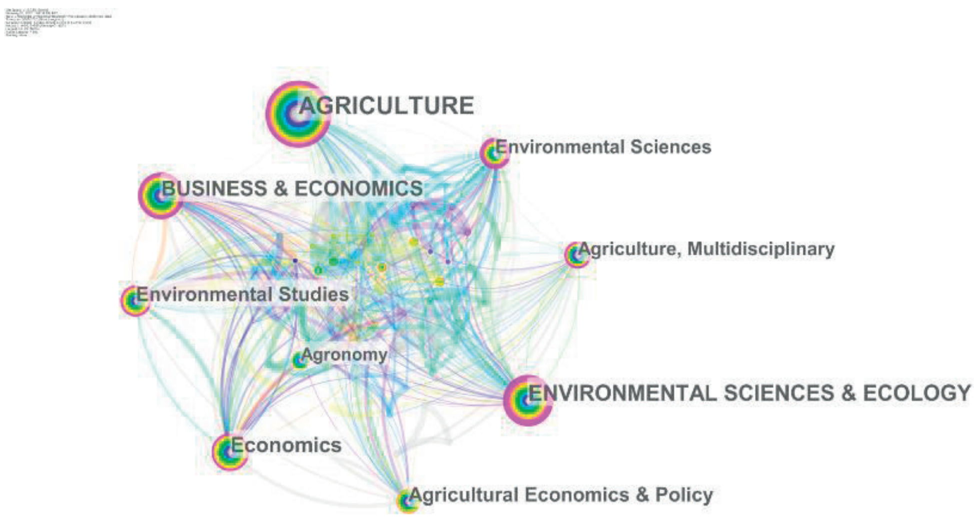

Figure 4. The category network between 2007 and 2020
(Source: CiteSpace platform)

Table 3 highlights the main categories taking into consideration the Frequency (Count) and the Centrality.

Table 3. The centrality of the categories

Count	Centrality	Year	WoS Categories
103	0.41	2007	ENVIRONMENTAL SCIENCES & ECOLOGY
160	0.31	2007	AGRICULTURE
90	0.24	2007	BUSINESS & ECONOMICS
53	0.21	2007	Environmental Sciences
76	0.18	2007	Economics
53	0.17	2007	Agriculture, Multidisciplinary
68	0.16	2007	Environmental Studies

Source: CiteSpace platform

From the perspective of the Frequency (citation counts), the main categories are Agriculture (160), Environmental Sciences & Ecology (103) and Bussiness & Economics (90). Studies about the modernisation of the agricultural holdings in the context of the Common Agricultural Policy were published in many journals as we can see in Figure 5.
Table 4 presents a situation of the number of citations.
For the first position, we have a “no title journal”, with over fifty citations.

We probably have this kind of situation because there are many citations from papers which do not belong to any journal.
The second-ranked journal in citations is the “World Development” journal with 45 citations and a centrality of 0.15, the same as in the first case. On the third position, we have the journal “Land use policy” with 42 citations, having a weaker centrality of about 0.06.

Table 4. Top 10 journals by frequency and centrality

Count	Centrality	Year	Cited Journals
52	0.15	2007	[NO TITLE CAPTURED]
45	0.15	2007	WORLD DEV
42	0.06	2008	LAND USE POLICY
39	0.16	2008	AGR SYST
36	0.14	2009	FOOD POLICY
32	0.06	2012	J RURAL STUD
31	0.10	2009	AM J AGR ECON
31	0.11	2007	AGR ECOSYST ENVIRON
26	0.08	2011	J ENVIRON MANAGE
26	0.21	2007	AGR WATER MANAGE

Source: CiteSpace platform.

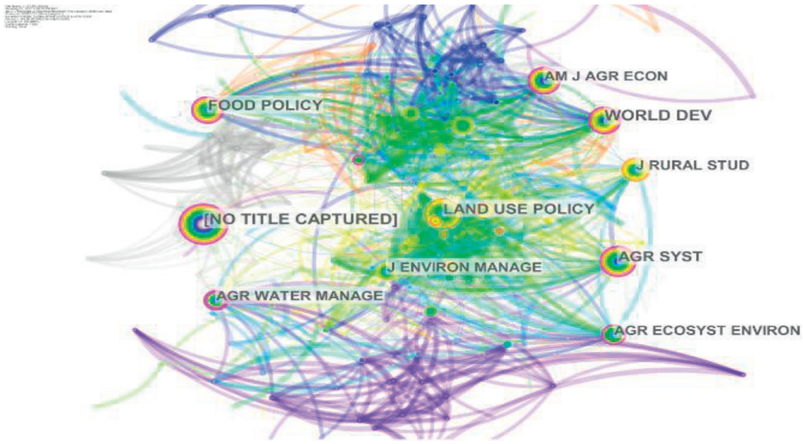

Figure 5. The network organized by the cited journal
(Source: CiteSpace platform)

The key words are viewed as of the most important points of a publication and co-words analysis can be used to discover research topics, analyze research hotspots, and monitor research frontier transitions from a knowledge domain (Yu, 2017).
In Figure 6 there are many key words links to our search like „agriculture”, „modernization”, „farm”, „impact” and others.

Table 5 presents the top 10 key words with the frequency and centrality that appear in the studies.
The most central key word and the one that the most frequently appears in the literature is agriculture with a frequency of 37 and a centrality of 0.41.

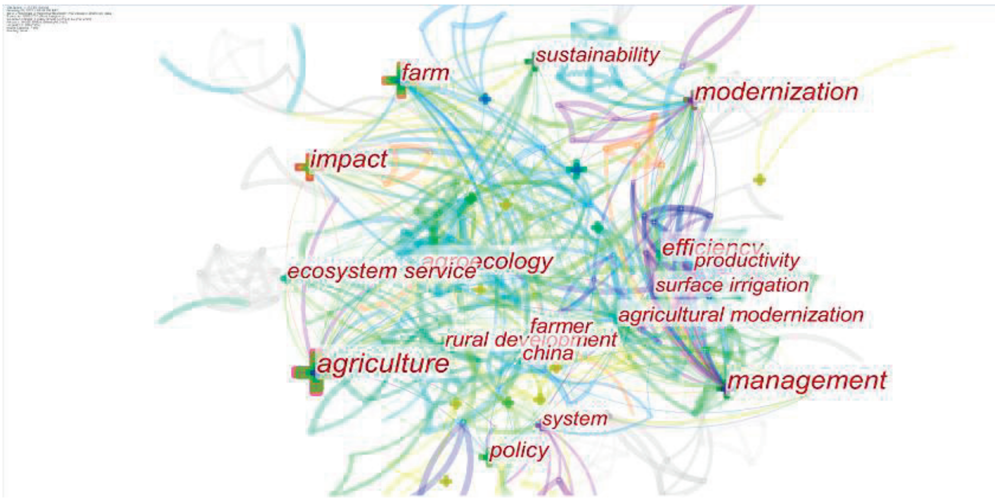

Figure. 6 The network organized by the cited key words
(Source: CiteSpace platform)

Table 5. Top 10 key words in terms of frequency and centrality

Count	Centrality	Year	Keywords
37	0.41	2007	agriculture
16	0.20	2009	management
14	0.22	2011	modernization
13	0.16	2008	sustainability
12	0.04	2013	farm
11	0.16	2013	impact
10	0.08	2007	policy
10	0.08	2008	efficiency
8	0.04	2011	system

Source: CiteSpace platform

Figure 7 exposes the main clusters labeled by the key words and Tabel 7 confers us the details of these clusters, where the size of the set is the number of terms included in each set.

In a short sentence, a cluster is a group of closely-coupled documents outlining different directions of research. From the topics of this group of documents, representative terms were selected and labeled by a log-likelihood ratio, expressed as "# þ Cluster ID þ representative term"(Chen, 2009).

As we can observe in Table 6, the silhouettes in our clusters are from 0.791 to 0.998.

One of the most relevant values, which impacting the overall structural properties of the network is the silhouette of scores. The homogeneity of a cluster is represented by the silhouette, ranging from -1 to 1 and the highest possible value 1 represents a perfect solution. (Chen, 2006).

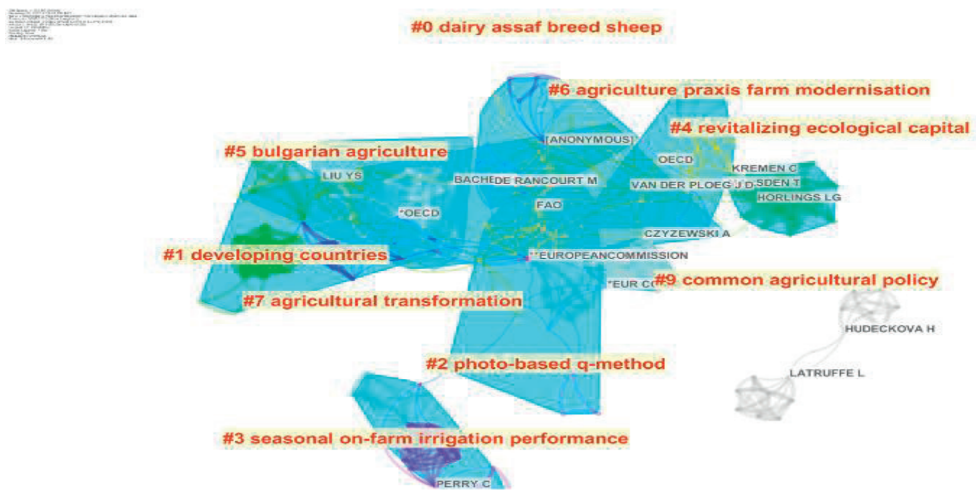

Figure 7. Main clusters labeled by key words in the field of farm modernisation
(Source: CiteSpace platform)

We have more consistent cluster members, with a more higher silhouette number. A good variety of clusters would-be about 7-10 major clusters with 10 or more members, with eachof the clusters having high silhouette values (e.g. > 0.70) (Chen, 2015).

Table 6. Summary of the 10 largest clusters, showing size, silhouette

Cluster ID	Size	Silhouette	mean(Year)
0	32	0.927	2009
1	32	0.941	2010
2	31	0.872	2012
3	24	0.988	2007
4	23	0.978	2010
5	22	0.955	2005
6	21	0.791	2011
7	20	0.806	2013
9	16	0.985	2007
10	12	0.998	2002

Source: CiteSpace platform

CONCLUSIONS

This paper provides a short review of the existing literature on the Web of Science Core Collection database in the subject of farm modernisation in the context of the Common Agriculture Policy.

By using scientometric methods throughout CiteSpace software we have abstractly pre-sented some indicators. The research in this field has grown over the years, representing an interesting research topic for researchers in the USA and the Republic of China. World Development and Land Use Policy Journals are among the most published sources. Regarding the main categories, "Agriculture", "Environ-mental Sciences & Ecology" and "Business & Economics" are on the top from the perspective of frequency and centrality. A weak link can be visible between the key word's advanced search using CiteSpace and the cluster view. However, words like "agriculture", "farm" and "modernisation" are the link between them, being essential keywords in our research.

Though, the present analysis of the bibliography provided by the Web of Science database does not furnish us with a very rich basis for our research.

Future research can be done in the respect of mapping the important literature regarding the subject of farm modernisation in the current context of the Common Agricultural Policy, by using other scientific databases (Scopus, Science Direct etc.).

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EVOLUTION OF NOSEMOSIS IN THE APIARY: INFLUENCE OF THE SEASON AND BEE TECHNOLOGIES

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Abstract

Bees and bee technologies are highly depending on the environmental factors. The seasonal dynamics of the bee colony are influenced by the both, bee technologies and quality and quantity of the nectaro-pollenifer sources. It is known that the impact of losses through bee mortality and colony unification (26.09%) due to the evolution of nosemosis are almost equal with the losses considered normal for the winter period, respectively of maximum 30%. The present study is based on observations carried out on the evolution of nosemosis during of three consecutive years (September, 2016 - March, 2019) in a stationary apiary in Romania. Investigations for the presence of Nosema spores in both living and dead bees were performed. Additionally, the presence of Nosema spores in honey, inside of the nests, before and after extraction was determined. The evolution and involution of the bee colonies were monitored according to the level of Nosema infection, by laboratory analyses on dead bees, especially during the first period of winter. The results clearly emphasized that, due to the presence of Nosema spores in the hive, in order to reduce the infectious pressure, it is imposing that in the spring the frames on which the bees have wintered to be reformed, even though they still have food resources (honey and bee-bread). Additionally, the breeding of bee colonies is recommended to be performed using only colony without the frames with food or frames from which hatched the brood, in order to avoid infection of the new colony.

Key words: *honey bees, nosemosis, apiary, Romania.*

INTRODUCTION

Nosemosis is a multifactorial pathological condition, endemic, without easily detectable clinical expressions (Bailey, 1955; Zander, 2009). To date, two microsporid species (Microspora: Microsporidida) have been described parasitizing honey bee: *Nosema apis* and *N. ceranae* (Fries et al., 1996; Chen et al., 2009). The main characteristic of the endemic diseases is the constant presence within an areal in different forms of the infectious agent. The endemic evolution of the disease is favored apart from the infectious reserve or pressure and by intrinsic factors (natural defense system, receptivity, homeostasis, caste, age etc.) and extrinsic (colony evolution according to the season, development conditions depending on the contribution nectar and pollen, climatic factors, colony power and applied technologies, stress factors etc.) (Fries, 1993; Mitrea, 2011; Gardi et al., 2015). The evolution of the disease can also be influenced by associated risk factors (associated diseases, poisonings, pest attack, uncontrolled propagation, loss of queens etc.). As spore reservoirs at the level of the bee colony, the relative importance of faecal contamination, honey and sheep reserves and bee carcasses are not fully clarified and understood. Without performing bee technologies, without veterinary (clinical and laboratory) health surveillance and without analyzing epidemiological indices (prevalence, incidence, mortality) we cannot control the evolution of the disease (OIE, 2013). The rapid and insidious development of the disease contributes to the underestimating severity of *Nosema* infection and its economic importance.
The aims of this study were to investigate the occurrence and evolution of nosemosis and to

estimate the infectious pressure in the hives, in a stationary apiary in Romania.

MATERIALS AND METHODS

The present study is based on observations carried out on the evolution of nosemosis during of three consecutive years (September, 2016 - March, 2019) in a stationary apiary, including 21 bee colonies, in Prahova county, Romania. Investigations for the presence of *Nosema* spores in both living and dead bees were performed. Additionally, the presence of *Nosema* spores in honey, inside of the honeycombs, before and after extraction was determined. The evolution and involution of the bee colonies were monitored according to the level of *Nosema* infection, by laboratory analyses on dead bees, especially during the first period of winter.

Veterinary sanitary surveillance of bee colonies from the hive taken in the study-exploited stationary apiary was done through clinical, morpho-pathological and laboratory investigations, depending on the season and the physiological status of the colony. The evolution of the bee colonies was followed by individual observation sheets and the applied technologies (revision, extraction, propagation, treatments) were recorded in the bee-hive book (Chioveanu, 2009; Fries et al., 2013; Dumitru et al., 2017).

The laboratory investigations aimed to identify the colonies of bees infected with Nosema spores and to determine the level of infection and the infectious pressure in the colony both on the bees (living or dead) and in the food reserves. Identification of spores in the laboratory was done according to OIE standards (OIE, 2013). For this purpose, two analytical methods were used, namely: method of identifying the *Nosema* spores by direct microscopic examination and a quantitative method of determining the number of *Nosema* spores. The samples were analyzed at the National Reference Laboratory for Honey Bees Health in IDAH (Chioveanu et al., 2009).

To identify *Nosema* spores in bees and to determine the level of infection, samples of at least 60 bee abdomens were processed.

To identify the spores of *Nosema* spp. in food reserves, samples of 5 g of honey / nectar from the frames were collected before and after extraction. The food reserve sample was mixed with distilled water in a 1:1 ratio, homogenized very well and filtered. The resulting filtrate was centrifuged for 6 minutes at 800 g and 25°C. After centrifugation, the supernatant was removed and the deposit was resuspended in 10 ml of distilled water, very well homogenized and samples were immediately microscopically examined on slides covered with cover slip, for detection of *Nosema* spores, accordingly the OIE standards.

A positive sample was considered when at least one *Nosema* spore was detected in at least one microscopic field. When the number of spores exceeds 9,000,000 spores/bee, the lesions in the anterior (ventricular) and middle (small intestine) intestines were considered irreversible, therefore the diagnosis of nosemosis was established (Chioveanu et al., 2009). In order to determine the number of spores/bees (Z) and implicitly the infection level, the calculation formula is used: $Z = \alpha 1 + \alpha 2/2 \times \delta \times 250{,}000$ where α represents the average of the readings from 10 microscopic fields and δ is the dilution factor. Unit to be analysed represents the average number of spores determined after reading two subsamples ($\alpha 1 + \alpha 2$), each 10 ml of triturate of bee abdomens or honey/nectar suspension. To determine the number of spores/bees, the resultant number / unit is multiplied by a constant characteristic for the Burker Turk hemocytometer counting chamber, which is 250,000.

RESULTS AND DISCUSSIONS

In order to investigate the incidence of nosemosis as an enzootic disease in the studied apiary (with 21 colonies) and to identify the sources of infection during the first period of the biopause (January 2017), live bees were collected and were subjected for determining the number of spores per bee. Subsequently, the degree of contamination was determined. The results are presented in Table 1.

The results show that during the first period of wintering the disease does not occur (nosemosis) in the apiary because there is a low level of infection, 19.05% of colonies being positive, with a burden of *Nosema* spores $\geq$ 5,000,000 spores/bee) (Figure 1).

Table 1. Data on *Nosema* infection of bees in a stationary apiary (Prahova county, Romania), in the first period of winter 2016-2017 (data from exams on live bees collected in January, 2017)

No. spores/unit	No. spores/bees	No. colonies	Percentage (%)
0	0	17/21	80.95
> 10	< 2,500,000	3/21	14.29
11-20	2,500,000-5,000,000	1/21	4.76

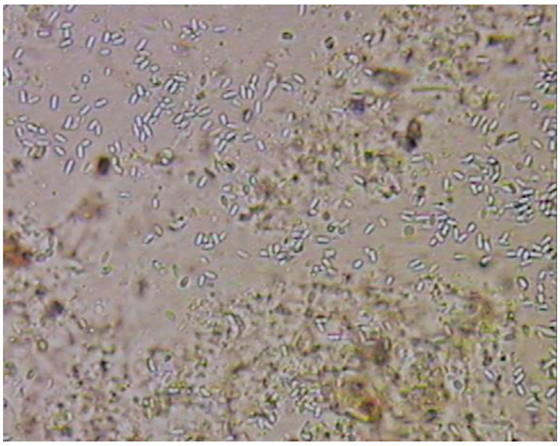

Figure 1. Masive *Nosema* spores infection (> 9,000,000/unit) (x400)

In the temperate zone, the biological evolution of the bee colony is in total dependence on the succession of seasons. Biopause - the first stage of winter rest - lasts 2-3 months when the bees are trapped in the winter hive and apparently inactive. Only in the second part of the winter, the colony intensifies its activity (the oviposition of queen starts, the consumption of food begins to increase, and the temperature in the hibernation increases from 8-10°C to 34-35°C). In early winter, spores are rarely encountered or are found only in the case of a very severe infection of bees. Most hives are supposed to contain spore-carrier bees that under the influence of extrinsic and intrinsic factors can develop the disease (Doull, 1961). Any inherent natural defense of a bee colony against a severe infection depends on the colony as well as the prevailing weather conditions in the first part of the autumn of the previous year (Steche, 1985). If these conditions are unfavorable, the overall life expectancy of the colony is reduced. This can lead to premature death of bees during winter or early spring.

For the second year included into the study (winter 2017-2018), dead bees collected from the bottom of the hive during the first period of winter, were tested and the results are presented in Table 2.

Table 2. Data on *Nosema* infection of bees in the apiary, in the first period of winter 2017-2018 (data from exams on dead bees collected in January, 2018)

No. spores/unit	No. spores/bees	No. colonies	Percentage (%)
0	0	8/23	35.00%
under 10 spores/unit	< 2,500,000	3/23	13.04%
11-20 spores/unit	2,500,000-5,000,000	4/23	17.39%
21-30 spores/unit	5,000,000-7,500,000	3/23	13.04%
31-40 spores/unit	7,500,000-10,000,000	3/23	13.04%
41-50 spores/unit	10,000,000-12,500,000	1/23	4.34%
over 50 spores/unit	>12,500,000	1/23	4.34%

In the beekeeping year 2017-2018, in the first wintering period, the results from the examination on dead bees collected from the bottom of the hive, showed that 65.20% of colonies were positive, emphasizing a very high infectious pressure. Overall, a mortality rate of 26.09% due to the evolution of nosemosis in the apiary was registered. The consequences of this evolution are presented in Table 3.

Table 3. Consequences of *Nosema* infection on the apiary (data from spring, 2018)

ID colony	No. of *Nosema* spores/unit	No. of *Nosema* spores/bee	Clinical observations	Consequences
3629	28	7,000,000	Death of all bees	Loss of colony
R300	45	11,250,000	Death of all bees	Loss of colony
3623	19	4,750,000	Depopulation	Unification
3620	18	4,500,000	Depopulation	
3618	8	2,000,000	Depopulation	Unification
R100	35	8,750,000	Depopulation	

The unification of colonies affected by the disease maintains subclinical nosemosis in the apiary. In the unified colonies there is a slow development, stagnation in evolution, depopulation and even a lack of production. Depopulation in hive by loss of the worker bees continues especially in autumn. In this case, the mortality rate registered (26.09%) due to the evolution of nosemosis and as a result of the unification of the affected families fall within the considered normal losses for the winter period, respectively of maximum 30%, as shown in the literature (Fries, 1993). Therefore, it could be neglected by the beekeeper and subsequently the true prevalence of nosemosis to be underestimated, by the lack of awareness and request for laboratory investigations.

All these findings could be explained also by the maintenance of old frames in hives. It is known that maintaining the bee colonies on old frames increases the risk of disease appearance (Frirs, 1988). In Europe the replacement of old frames is an important sanitary measure (Jordan, 1960; Zdra 1964). The old frames also have a negative influence on the quality of honey (Bailey, 1955b; Baer, 1964; Irsunov, 1982) and allow the spores to accumulate.

To investigate the presence of *Nosema* spores in honeycombs, fragments of honeycombs, from three colonies, were collected before and after honey extraction, and analyzed (Figure 2). The results are presented in Table 4.

Figure. 2. Frames and honeycombs before and after honey-extraction (clean)

Table 4. Presence of spores of *Nosema* spp. in honeycombs in the investigated apiary (three colonies were investigated)

ID Colony	Honeycombs before extraction		Honeycombs after extraction		Live bees	
	Sp/5 g product (no.)	Sp/unit (millions)	Sp/5 g product	Sp/unit (millions)	Sp/unit (no.)	Sp/bee (millions)
3632	67.6	16.9	62.8	15.7	3	0.75
R200	51.5	12.9	58.7	14.7	1	0.25
9523	23.2	5.8	28.2	7.1	8	2.0

The results showed a high level of Nosema spores in honey combs (varying from 23.2 to 67.6 spores per 5 grams of product). This could be a significant risk factor for outberack of acute diseases, in correlation with concurrent factors such as stress, unfavourable climatic factors.

Therefore, it is confirmed that maintaining the infectious pressure of *Nosema* spores in the bee colony is favoured by the breeding and exploitation technologies applied by the beekeeper. Contamination and maintenance of infectious pressure in hive are realized by exchanging honeycomb frames with brood or exchanging brood between colonies. If honey frames are contaminated with *Nosema* spores and are re-introduced into the hive after honey-extraction, the bees can be contaminated.

As can be seen in the Table 4, after the introduction of the contaminated honeycombs, the laboratory analyses confirmed the presence of *Nosema* spores in the body of live bees.

Additionally, crushing the sick bees during various activities, feeders and stagnant watering sourses with sick bees, all can favour the contamination of healthy bees. Existing honey and pasture reserves can exacerbate the disease and lead to the depopulation of the bee colony.

To investigate the impact of the re-use of infected frames (after honey extraction), the dynamics of infection was determined, by analyses on live and dead honey bees from the three colonies included in the study (Table 5).

Table 5. The dynamics of nosemosis in working bees in colonies in which frames were re-introduced (after honey extraction)

Date	Type of analyzed sample	Number of spores/unit		
		Colony 3632	Colony R200	Colony 9523
29.01.2018	Live bees	nd	nd	nd
	Dead bees	31	0	0
23.05.2018	Live bees	0	0	0
	Dead bees	3	nd	nd
08.06.2018	Live bees	3	1	8
	Dead bees	51	32	13
29.06.2018	Live bees	1	58	21
	Dead bees	nd	nd	nd

Legend: nd = not determined

After introducing in hive frames from which honey was extracted, we identified up to 58 spores of *Nosema* per unit (in living or dead bees). So, it is known that contaminated honey and bee bread can exacerbate the disease and depopulate the bee colony.

Old frames and honeycombs with food reserves represent source of spores, maintaining the infectious pressure within the hive. Therefore, in order to reduce the infectious pressure, in the spring, the frames on which the bees have wintered must be reformed, the honey and the remaining bee bread during the winter must be removed to avoid to be consumed by bees.

Additionally, the breeding of bee colonies should be done with new bee-packs and new frames, avoiding re-using of frames with food reserves or frames from which the bee brood hatched.

Based on the registered data it has been observed that the temporary dynamics of Nosema infection in the investigated apiary was characterized by a slow increase of the infection during the winter, with the worsening in the spring associated with the beginning of the brood growth, a low prevalence in summer, and a small peak in autumn.

Subsequently, it is emphasized the importance of the correct diagnostic of disease, including the infectious pressure at the colony level, the spreading of infection within the apiary, and the dynamics of infection during the year following a well-defined and standardized protocol for the both sampling and diagnostic (Chioveanu et al., 2009; Fries et al., 2013; OIE, 2013). Additionally, for the best health of the bee colonies, other indicators should be monitored: the number of dead bees in the first period of wintering; unhatched brood; detritus; microbial and fungal load of honey crowns.

Also, adequate pastures with attractive vegetation for the honeybees would help to increase their population, with subsequent benefits for the both farmer economy and ecosystems (Gardi et al., 2015).

The sanitary-veterinary surveillance should be done accordingly to the season and the physiologic status of the bee colony (Webster et al., 2004; Papini et al., 2017). Moreover, further investigations to establish the species of *Nosema* that are currently of high concern, are also planned for the near future.

CONCLUSIONS

The present study emaphasizes the evolution of nosemosis in a stationary hive in Romania, with seasonal features and also the influence of different technology actions in maintaining and/or exacerbating the disease, with serious consequences, including depopulation.

Therefore, maintaining the stability of the bee colony through modern technologies and interventions as little as possible ensures its homeostasis. In case of nosemosis, similarly as in other endemic diseases, the main actions must refer to the reduction of infectious pressure by applying specific prevention and control measures.

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RESEARCH REGARDING THE APPROACH OF DROUGHT TOLERANCE OF TWO-ROW SPRING BARLEY

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Abstract

Drought is one of the abiotic stress factors which affects the global production of grain cereals and is an important risk factor even for consecrated cultivation sites. This is even more acute for spring grains which are the most vulnerable to drought. This is the case for two-row spring barley which is highly sensitive to heat, especially in the post-anthesis phenophase. To monitor drought tolerance, we assess a number of 27 genotypes from the germplasm collection to identify the tolerant ones, to turn them into valuable genitors for cultivars adaptable to the new climate changes. In order to evaluate drought tolerance was used the principle of inducing this phenomenon through the use of chemical desiccants ($NaClO_3$). The method is based on sprinkling the plants with desiccants ($NaClO_3$) with a 2% concentration, 14 days after the anthesis. The genotypes that constitute the objective of this study are analysed through the prism of some components of production that are most significantly affected by the effects of drought appearing in the post-anthesis phenophase, such as grain weight/spike and thousand grain weight (TGW). The most pronounced effect of the treatment is highlighted by the case of the genotypes Concerto and Vienna which reacts very favourably.

Key words: *drought tolerance, grains weight, TGW, two-row spring barley.*

INTRODUCTION

Of all the abiotic stress factors that reduce crop productivity, drought is the most devastating and more resistant to the efforts of breeders (Tuberosa and Slavi, 2006). The complexity of this factor is determined by the number and diversity of the factors involved in plant response to stress.

From a practical point of view, drought tolerance is related to final yield rather than to plant's ability to survive under water-poor conditions (Tuberosa and Slavi, 2006). On the other hand, it can also be described as a culture's ability to maintain constant yield, regardless of environmental conditions, a concept known as yield stability (Cattivelli et al., 2002).

There are studies that reveal that in case of wheat, an increase in the average temperature by 1°C above the optimal value has caused a 3-4% reduction in yield, worldwide. In this context, even when water is not a limiting factor, the yields of cereals that go through high temperature periods towards the end of the growing season have lower yields, especially due to the negative impact of heat during the grain filling period (McDonald et al., 1983; Tewolde et al., 2006).

Development of wheat and cereal grains in general is influenced by: carbohydrates produced by the anthesis and translocated directly into the grains; carbohydrates produced after anthesis but temporarily stored in the stalk, then remobilized to the grains; carbohydrates produced before the anthesis, stored mainly in the stalk and remobilized to the grains during the filling of grains (Ehdaie et al., 2006).

Regarding the critical period for growing and filling of grains, usually in June, the year 2017 distinguish by increases of average monthly temperatures by 2.8°C and 54.2 mm deficit of rainfall compared to the multiannual average. Contrary to 2017, the year 2018 records closer values to thermal and rainfall normal level, with temperature increases of 1.5°C and rainfall increases of 3.4 mm compared to multiannual values.

MATERIALS AND METHODS

A total of 27 genotypes were selected from the two-row spring barley collection, which is represented by valuable genitors commonly used in breeding work:

- 9 genotypes of native origin (5 from ARDS Turda and 4 from ARDS Suceava);
- 18 genotypes of Western European origin (Germany, Holland and England) and Eastern European origin (Czech Republic and Estonia).

The ability to use stored reserves in the stalk for grain filling when the photosynthetic source is completely inhibited by stress factors can be assessed by destroying the photosynthesis source at the beginning of the grain filling period. It is determined the weight of grains in the absence of a current photosynthesis compared to the untreated control (Blum, 1998). Treating plants with oxidizing chemicals, such as sodium chlorate ($NaClO_3$), has the effect of destroying the photosynthetic device. This method was proposed by Blum et al. (1983). The treatment is applied by spraying the entire plant or just the flag leaves and the spike, two weeks after the anthesis, taking as reference the heading date. In parallel, untreated plants are kept as controls.

The study is presented like a polyfactorial experience of A×B×C type, where: A factor is the experimental year, with two graduations (2017, 2018); B factor is the treatment, with two graduations (untreated and treated); C factor is the genotype (with 27 varieties).

Following the biometric measurements, the rate of grain weight reduction (r) was calculated using the below formula:

$$r = \frac{(untreated\ grain\ weight - treated\ grain\ weight)}{untreated\ grain\ weight} \times 100$$

RESULTS AND DISCUSSIONS

The weight of grains/spike is one of the most important components of yield; the analysis of this trait is presented in Table 1. Under normal conditions are remarked for high grain weight/spike (greater than 1.45 g) the following genotypes: Turdeana, Daciana, Jubileu, Romanița, Prima, Adriana, Bogdana, Vienna, Alexis, Scarlet, Elisa, Concerto, Sulilly, Salome, Emir Swabeth (for 2017) and Turdeana, Daciana, Romanița, Adina, Bogdana, Scarlet, Concerto, Emir Swabeth (for 2018), along with the Belgravia genotype that is particularly evidenced by the stability of this trait in both experimental years. Under the influence of the stress factor are remarked the genotypes: Turdeana, Romaniţa, Prima, Adriana, Bogdana, Vienna, Victoriana, Alexis, Scarlet, Elisa, Concerto, Sulilly and Salome in 2017 and Belgravia and Concerto in 2018, all of them register values of the grain weight above 1.3 g/spike.

Relative values for the rate of grain weight reduction by using the desiccant register a significant variation in both genotypes and between the two experimental years, thus significant oscillations of both the minimum and maximum values from one year to the next suggests the major involvement of the environment in the control of the grain weight/spike. Regarding the fluctuations in the rate of grain weight reduction, it can be said that there are obvious differences between genotypes under the influence of stress factor.

The genotypes Adina, Victoriana, Salome and Concerto are noted for a low rate of grain weight reduction for 2017 experimental year, and the genotypes Chronicle, Salome, Concerto and Victoriana for 2018.

Most of the breeders' beliefs lead to the idea that the grains weight is a character of low heritability, so that the phenotypic expression of this trait can be greatly influenced by both climatic and technological factors. But taking into account that the same cultivation technology is used in the breeding field, the differences between genotypes in the two experimental years regarding the averages and the coefficients of variation under normal conditions, would be due to climatic factors (Table 1).

At the same time, it can be noticed that at the level of the 27 genotypes there is a small to moderate variability, the values of the coefficients of variation oscillating from 8.20 to 12.51 (Table 2).

Higher values of the variation coefficients under the influence of treatment indicate a greater fluctuation of the values around the average relative to normal conditions.

Table 1. Genotypes behaviour on grain weight/main spike (g) and the reduction rate

Cultivars	Untreated		Treated		Reduction rate (%)	
	2017	2018	2017	2018	2017	2018
Turdeana	1.58	1.53	1.36	1.25	14.04	18.21
Daciana	1.72	1.60	1.25	1.19	27.00	26.03
Capriana	1.38	1.21	1.02	0.84	26.33	30.40
Jubileu	1.52	1.34	1.25	1.25	17.45	6.65
Romanița	1.69	1.65	1.43	1.10	15.01	33.32
Prima	1.53	1.37	1.40	1.07	8.38	21.80
Farmec	1.29	1.25	1.10	1.04	14.57	16.55
Adina	1.62	1.50	1.58	1.20	2.13	20.45
Bogdana	1.60	1.53	1.40	1.26	12.57	17.88
Sidney	1.40	1.28	0.92	1.11	33.98	13.24
Steward	1.42	1.14	1.16	1.05	17.94	7.48
Mauriția	1.38	1.40	1.22	1.21	11.09	13.09
Marlen	1.42	1.36	1.27	1.23	10.29	9.43
Vienna	1.57	1.41	1.42	1.26	9.32	10.38
Victoriana	1.42	1.21	1.35	1.11	4.78	8.62
Marthe	1.28	1.23	1.07	1.11	15.88	9.52
Alexis	1.58	1.20	1.39	1.06	12.31	11.58
Scarlet	1.66	1.45	1.44	0.95	13.12	34.33
Jubilant	1.41	1.12	1.10	0.99	22.24	11.71
Elisa	1.48	1.30	1.43	0.90	3.49	30.87
Chronicle	1.43	1.22	1.29	1.21	9.38	0.32
Belgravia	1.61	1.61	1.29	1.49	19.43	7.43
Concerto	1.68	1.48	1.52	1.35	9.50	8.47
Sulilly	1.51	1.15	1.18	1.13	21.59	1.73
Salome	1.46	1.22	1.36	1.15	6.92	5.75
Arupo	1.35	1.21	1.09	1.04	19.40	13.80
Emir Swabeth	1.60	1.48	1.29	1.27	19.15	14.62

Table 2. Analysis of variability parameters for grain weight/spike (g)

Year / Parameter	2017		2018	
	Untreated	Treated	Untreated	Treated
Average	1.50	1.28	1.34	1.14
Standard deviation	0.12	0.16	0.16	0.14
Amplitude	0.44	0.66	0.53	0.65
Minimum	1.28	0.92	1.12	0.84
Maximum	1.72	1.58	1.65	1.49
Variation coefficient (%)	8.20	12.51	11.77	12.30

Frequency of genotypes in terms of reduction rate for grains weight on the main spike indicates their grouping predominantly in the range of 5.3-20.3%. It can also be mentioned that there is a fairly pronounced analogy in the two experimental years on the response of genotypes to stress factor, so in 2017 the modal is between 10.34-15.34%, and in 2018 this is slightly shifted to the left to lower values between 5.33-10.33% (Figure 1).

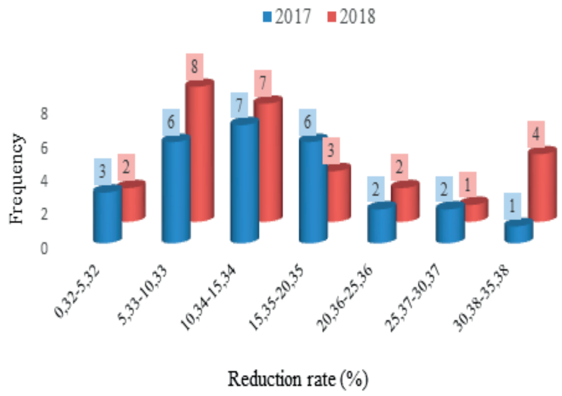

Figure 1. Frequency of genotypes for the reduction rate of grains weight

Drought tolerance can also be seen as a skill of genotypes to maintain yield parameters as stable as possible from year to year and with the highest performance.

In this context, for the breeding process the valuable sources are represented mainly by the genotypes which in both experimental years exhibit reduced fluctuations in the reduction rate of grains weight. Thus, a series of genotypes placed in the first quadrant were identified, which in both experimental years recorded values below the reduction rate average and above the average grains weight.

These can be considered genotypes that have a pronounced stability under the influence of the treatment or action of abiotic stress factors (drought).

Consequently, the genotypes Concerto, Vienna, Chronicle, Marlen and Mauritius (Figure 2) are particularly noteworthy.

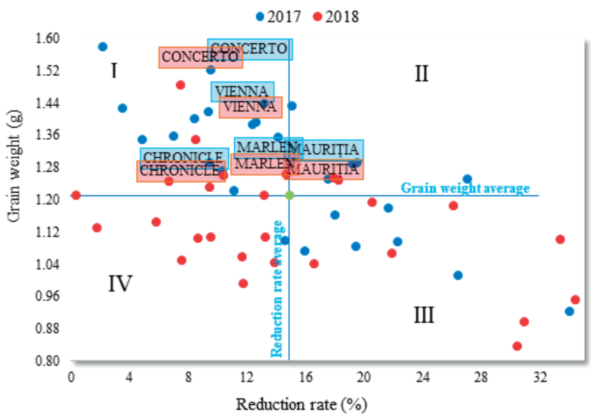

Figure 2. Stability of genotypes for grain weight reduction rate and grain weight

Even though the breeders often do not give up yield in favour of stability, yet under the current conditions where the climatic years are very different, the stability of the crops receives a new dimension in the breeding programs. Given the current increases of annual average temperatures, it is important to identify genotypes capable of fulfilling these requirements and to include them in various breeding schemes.

Thousand grain weight (TGW) trait is one of the main qualitative parameters of cultivars intended to beer industry. TGW must exceed the admissible minimum of 42 g.

Thus, a higher value of this parameter is directly correlated with higher starch content and superior germination energy. The highest values of TGW, both treated and untreated, are recorded by Belgravia genotype followed by Concerto, and in the normal conditions are also noted the native genotypes Daciana and Romaniţa (Table 3).

Table 3. Genotypes behaviour on thousand grain weight (TGW) and the reduction rate

Cultivars	Untreated		Treated		Reduction rate, %	
	2017	2018	2017	2018	2017	2018
Turdeana	54.04	50.87	45.15	40.97	16.44	19.47
Daciana	58.82	54.82	42.95	40.54	26.98	26.06
Capriana	50.65	46.89	40.99	36.73	19.07	21.67
Jubileu	51.81	52.54	46.22	47.27	10.78	10.02
Romanița	55.62	58.96	45.95	42.54	17.39	27.85
Prima	54.08	54.53	49.22	41.06	8.99	24.70
Farmec	50.19	50.18	41.04	43.37	18.23	13.57
Adina	56.16	53.93	54.77	43.05	2.48	20.17
Bogdana	53.61	55.24	46.75	47.22	12.79	14.51
Sidney	57.43	59.45	38.57	46.89	32.83	21.13
Steward	54.47	51.58	44.95	46.64	17.48	9.57
Mauriția	53.73	55.87	45.65	49.39	15.03	11.59
Marlen	52.21	50.32	49.58	43.65	5.05	13.26
Vienna	58.04	53.37	50.92	46.11	12.26	13.60
Victoriana	56.64	52.61	51.36	42.72	9.32	18.80
Marthe	51.74	49.18	45.78	46.39	11.52	5.67
Alexis	54.93	51.04	45.80	38.83	16.63	23.93
Scarlet	53.89	50.37	47.52	36.89	11.81	26.75
Jubilant	50.39	49.15	47.64	39.77	5.46	19.08
Elisa	53.38	51.38	46.76	47.21	12.41	8.12
Chronicle	53.90	53.16	45.42	52.95	15.74	0.38
Belgravia	59.87	65.91	54.54	62.67	8.91	4.91
Concerto	59.33	62.33	53.99	54.87	9.01	11.98
Sulilly	58.52	51.96	52.64	50.11	10.05	3.56
Salome	51.16	47.04	41.22	42.42	19.43	9.83
Arupo	47.50	45.96	43.19	37.02	9.08	19.46
Emir Swabeth	53.48	49.31	41.95	42.56	21.56	13.70

Most affected genotypes of the effects of induced drought that have a reduction rate over 15% are: Daciana (27%), Turdeana (16%), Capriana (19%), Romaniţa (17%), Farmec (18%), Steward (17.48%), Alexis (17%), Chronicle (16%), Salome (19%) and Emir Swabeth (22%). Some of these genotypes are also found in 2018 with high values of the reduction rate of TGW, namely Daciana, Turdeana, Capriana, Romaniţa, Sidney and Alexis. Therefore, it could be extrapolated that these genotypes have less power to translate assimilates from vegetative parts to grains. This assertion is also based on the fact that under normal conditions, some of these genotypes perform for this trait: Daciana (58.82 g in 2017 and 54.82 g in 2018), Romaniţa (55.62 and 58.96) and Sidney (57.43 and 59.45).

Although most breeders claim that this trait is closely related to genotype, the difference between averages over the two experimental years suggests that besides genotype, also environmental factors have influence on the phenotypic expression of this trait. It can also be noticed that by applying the treatment, the values of variation coefficients increase; that suggests the differential response of genotypes and a more pronounced degree of spreading of values around the average.

The useful portion of the variability that could lead to a plus value is between the average and the maximum values (Table 4).

Table 4. Analysis of variability parameters for TGW (g)

Parameter / Year	2017		2018	
	Untreated	Treated	Untreated	Treated
Average	48.45	46.69	52.89	44.81
Standard deviation	3.98	4.35	4.61	5.85
Amplitude	14.16	16.19	19.95	25.94
Minimum	41.20	38.57	45.96	36.73
Maximum	55.36	54.77	65.91	62.67
Variation coefficient (%)	8.20	9.31	8.71	13.05

Most genotypes record a TGW reduction rate below 20%. However, in 2018, seven genotypes register values above 20% compared to 2017 when only three of them exceed this limit. Differentiated expression of genotypes from year to year in the TGW reduction rate suggests that climatic conditions also play an important role in the differentiated rhythm of carbohydrate assimilation genotypes (Figure 3).

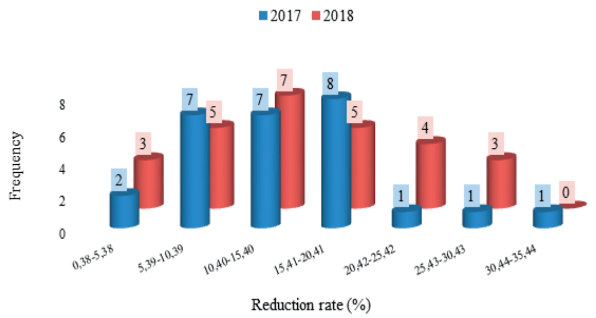

Figure 3. Frequency of reduction rate for TGW

From the perspective of TGW, the genotypes Belgravia, Concerto, Sulilly, Jubilee, Marthe, Elisa, Vienna and Bogdana can be considered genotypes with a pronounced stability under the influence of treatment, as in both experimental years they record values above the TGW average and below the reduction rate. Therefore, it can be argued that in these genotypes the rate of reduction is not influenced largely by the environmental conditions and therefore on these genotypes the transfer of assimilates to the grain is not disturbed by the environmental conditions (Figure 4).

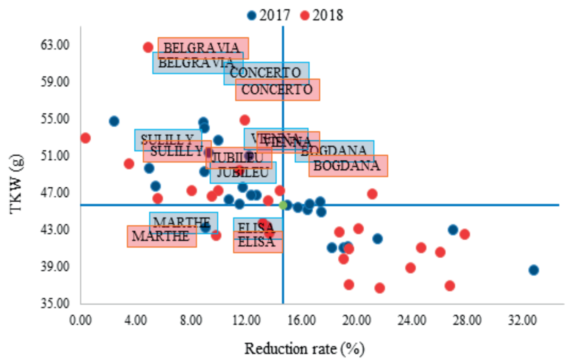

Figure 4. Stability of genotypes for TGW reduction rate and TGW

CONCLUSIONS

Concerto and Vienna varieties respond highly favourable to the influence of stress factor on the stability of grain weight on the main spike and of TGW, as well as the rate of reduction of these analysed traits, both under the conditions of a favourable 2017 year, but also under the less favourable 2018 year. Therefore, these two varieties can be considered as valuable genetic sources for the stability of both parameters under heat stress conditions during the post-anthesis period.

Generally speaking, under the stress conditions a significant percentage of genotypes respond favourably both in terms of grains weight and TGW. Thus, 18.52% is represented by the genotypes Concerto, Vienna, Chronicle, Marlen and Maurice, which under stress conditions record values of grain weight over the average of the two experimental years (1.21 g), but also with a reduction rate of grain weight below 14.83%. On the other hand, regarding TGW, a percentage of 22.22% is represented by the genotypes Belgravia, Concerto, Vienna, Sulilly, Jubileu, Bogdana, Marthe and Elisa, which record values above the average of 45.75 g and a reduction rate below the average of 14.63%.

Enlarging the identification area of valuable genotypes in terms of yield loss limitation under conditions of simulated atmospheric draught, constitutes an important prerequisite in order to create new varieties tolerant to this limitative factor which appears in the critical phenophase of grain filling. Also, the most valuable lines from the comparative cultures will be tested in this respect, too.

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THE PRODUCTIVITY OF SPRING BARLEY VARIETIES DEPENDING ON THE OPTIMIZATION OF NUTRITION IN THE SOUTHERN STEPPE OF UKRAINE

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Abstract

The article presents the results of research carried out in 2013 - 2017 in the conditions of the experimental field of the Mykolayiv National Agrarian University in southern chernozem to study the efficiency of the treatment of barley crops with modern growth-regulating preparations in the background of mineral fertilizers. The influence of variety (Adapt, Stalker, Aeneas) and ferilization ($N_{30}P_{30}$, Urea K1, Urea K2, Escort-bio, Organic D2) of barley plants on the formation of elements of the crop structure (total number of stemsand productive stems, number of grains in the ear and weight of grain from one ear) and the level of grain yield were investigated. It was determined that the application of preseeding cultivation of spring barley with mineral fertilizer at a dose of $N_{30}P_{30}$ (background) and the application of extra-root cropping at the beginning of the phases of stooling and the earing with fertilizers Urea K1, Urea K2,Organic D2 and Escort-bio created favorable conditions for the formation of optimal grain yield levels. It was determined that the highest yield of barley grain was formed in experimental variants using Organic D2 (3.22-3.56 t ha^{-1}) and Escort-bio (3.25-3.61 t ha^{-1}), depending on the variety. These exceeded the yield rate on an unfertilized plot by 20.5-21.3 and 21.2-22.4% for Organic D2 and Escort-bio, respectively, and with the application of mineral fertilizers only, the yield rate grew by 12.0-13.5%. The highest yield of grain was formed by plants of the Aeneas's variety: 2.80-3.61 t ha^{-1}, depending on the variants of nourishment.

***Key words**: spring barley, variety, plant nutrition, growth-regulating substances, crop structure, grain yield.*

INTRODUCTION

Grain production belongs to the strategically important branches of the agrarian economy in Ukraine. Since it provides a significant part of the income at the agricultural enterprises, it also occupies more than half of the area under cultivation and predetermines the constant demand and high demand of people for the consumption of food products. The grain is a raw material, is also an indispensable source for the establishing of complete forage base for the development of livestock industries (Kushniruk et al., 2016; Kozera et al., 2017).
The spring barley is one of the leading grain-harvesting crops in Ukraine and in the flat area, and the gross collection it takes the second place after the winter wheat. With a high potential grain yield of modern varieties (about 9.0 t ha^{-1}), the average level of barley yield remains low (because the precipitation is low during vegetation, non-compliance with elements of technologies), unstable with fluctuations within years under the influence of many factors - it varies up to 40% or more, which does not fully meet the needs of the national economy with food grain, fodder and brewer's grain (Kaminskaya et al., 2012; Kolesnikov et al., 2016). Growing barley in the zone of the Southern Steppe of Ukraine after a better predecessor (soybean), in the background of making integrated mineral fertilizers contributes to the yield of grain yield at the level of 4.03 t ha^{-1}. The sowing of barley after sunflower leads to a shortage of the yield by 15.4%, and the sowing of barley after winter wheat leads to a shortage of the yield by 7.7%. At the same time the application of fertilizer contributed to the growth of grain yield regardless of its precrop (it increased by 23.0-39.3%). At the same time, the profitability of spring barley growing after soybeans was 102.7%, and it after sunflower and winter wheat was 68.5 and 98.4%, respectively. At the same time, the level of profitability of cultivating the crop was 102.7% (Gyrka et al., 2014).

Improving the technology of spring barley growth is an extremely urgent task, since under the current economic conditions cheapening of grain production and increase of its profitability is possible only in the case of application of new agrotechnical methods which do not involve high costs. Modern intensification of crop production in the conditions of acute deficiency of organic fertilizers and too high prices for mineral fertilizers involves the development of alternative measures of technology of crop cultivating. In the context of this, the study of the influence of highly effective polymer chelate fertilizers, biopreparations, growth-regulating substances, etc. in combination with other agrotechnical elements on the formation of biometric indices of plants, productivity and quality of production becomes of increasing importance (Rozhkov et al., 2017). There is a need for the development and implementation of resource-saving elements in plant nutrition technology, which consists of applicating of low doses of mineral fertilizers and, on their background, using of extra-root nutrition with modern substances in the main periods of their vegetation (Gamayunova et al., 2017).

Plant nutrition during the vegetation period provides the reduction of plant stress caused by adverse weather and climatic conditions, which, according to many scientists, is an integral part of intensive agricultural production (Sepiedeh et al., 2014; Begum et al., 2015).

Mineral fertilizers, including increasing doses of nitrogen fertilizers, contribute to the growth of the biomass of plants and the increase in grain yield (Novotna et al., 2015; Povilaitis et al., 2018). One of the ways for increasing the effectiveness of mineral fertilizers, used for reducing their norms, is the use of growth stimulants. Due to synthetic fertilizers, the plant resistance to adverse weather conditions increases, also to the damage by pests and diseases, etc. According to the data of research, it was determined that the use of modern growth-regulating substances on grain and legumes (Fabaceae) crops is worth the cost of a yield increments (growth) for 30-50 times, and is in sunflower - for 50-100 times, in fact, this measure is one of the most profitable in yield raising (Panfilova et al., 2018; Kolesnikov et al., 2016).

Numerous studies of scientists in the world found that the use of complex organic fertilizers, composite biological growth-regulating substances, inoculants, nanoparticles, and nutrient elements contributed to the regulation of growth processes and development of plants, their resistance to stress through increased plant immunity, activation of biological processes, synthesis of organic substances, increasing the area of the leaf surface, the increasing in the net productivity of photosynthesis and the yield (Singh et al., 2018; Pestovsky et al., 2017; Piskaeva et al., 2017; Wakchaure et al., 2016; Klein et al., 2018). However, today, the market presents a very wide range of substances, which complicates their choice, and the number of scientific evidence regarding the impact of these substances on the productivity of barley in the world of scientific literature is not studied. Taking into account the acuteness of the problem, the aim of the study was to determine the impact of modern certified substances, in particular organo-mineral fertilizers Organic D2 and natural microbial complex Escort-bio, on the productivity of barley of spring varieties Adapt, Stalker and Aeneas, which involves the partial replacement of mineral fertilizers and chemical pesticides in order to increase the yield. The relevance of this study increases with the globalization of the influence of anthropo-technological load on the natural environment and the growth of the rate of depletion of natural ecosystems.

MATERIALS AND METHODS

Experimental researches were carried out during 2013-2017years in the location of the educational-scientific-practical center of the Mykolayiv National Agrarian University.

The soil of experimental sites was represented by the southern, resiliently weakly sunny, heavy-sooty black soilon the loesses. The reaction of the soil solution was neutral (pH 6.8-7.2). The content of humus in the 0-30 cm layer was 3.1-3.3%. The arable layer of soil contained moving forms of nutrients on average: nitrates (by GrandvalLiagou) as 15-25, mobile phosphorus (by Machigin) as 41-46, exchangeable potassium (on a flame photometer) as 389-425 mg/kg of soil.

The territory of the farm locates in the third agro-climatic region and belongs to the subzone of the southern steppe of Ukraine. The climate here is temperate-continental, warm, dry, with unstable snow cover. Weather conditions by hydrothermal indices during the research years varied, which gave an opportunity to obtain objective results. During the period of spring barley vegetation, the temperature of the air exceeded the average annual parameters by 0.3-1.4°C, depending on the year. The only exception was 2016, where the temperature of the air during the vegetation period was +14.9°C, which was somewhat lower than the long-term figures. During the vegetation of spring barley, depending on the year of the study, the precipitation fell as 95.8-189.5 mm. At the same time, in 2015 and 2016, the largest precipitation was 189.5 and 179.0 mm respectively, which exceeded the average annual figures by 15.1-19.8%.

The object of research was spring barley - varieties Adapt, Stalker and Aeneas. The technology of their cultivation, with the exception of the investigated factors, was generally accepted to the existing zonal recommenddations for the Southern Steppe of Ukraine. Weather conditions in the years of research varied, in particular, in 2015 and 2016 during the vegetation the considerably more rainfall dropped. In general, they were typical for the southern step peregion of Ukraine.

The total area of the experimental plot (the research work was organized by the random method of choosing the plots) was 80 m^2, the basic plot was 50 m^2 (length - 21.18 m, width - 2.36 m), repetition in the experiment was done three times. Pre-crops was sown peas *Pisum sativum* L. The scheme of the experiment included the following options:

Factor A - variety: 1. Adapt; 2. Stalker; 3. Aeneas.

Factor B - plant nutrition: 1. Control (without fertilizers); 2. $N_{30}P_{30}$- under pre-sowing cultivation - background (nitrogen was used in the form of ammonium nitrate (34% N), and phosphorus was in the form of double phosphorus (46% P); 3. Background + Urea K1 (1 l/ha); 4. Background + Urea K2 (1 l/ha); 5. Background + Escort-bio (0.5 l/ha); 6. Background + Urea K1 + Urea K2 (0.5 l/ha); 7. Background + Organic D2 (1 l/ha). The standard working solution was 200 l/ha. The fertilization of crops by fertilizers was carried out at the beginning of the phases of the spring barley stooling (BBCH 31) and earing (BBCH 51).

Preparations to be used for foliar application of barley crops were listed in the List of pesticides and agrochemicals authorized for use in Ukraine. Preparations of Urea K1 and Urea K2 are registered as fertilizers containing respectively N as 11-13%, P_2O_5 as 0.1-0.3%, K_2O as 0.05-0.15%, micronutrients as 0.1%, succinic acid as 0.1% and N as 9-11%, P_2O_5 as 0.5-0.7%, K_2O as 0.05-0.15%, sodium humate as 3 g/l, potassium humate as 1 g/l, trace elements as 1 g/l. Organic D2 is organo-mineral fertilizer containing N as 2.0-3.0%, P_2O_5 as 1.7-2.8%, K_2O as 1.3-2.0%, total calcium as 2.0-6.0%, organic matter as 65-70% (in terms of carbon). Escort-bio is a natural microbial complex that contains strains of microorganisms of genera *Azotobacter*, *Pseudomonas*, *Rhizobium*, *Lactobacillus*, *Bacillus*, and biologically active substances produced by them.

In the process of research, the method of the State Variety Testing of Agricultural Cultures was used (Volkodav et al., 2001). The sowing was done during the third ten-dey period of March, harvesting - the first ten-day period of July. The crop structure (total number of stemsand productive stems, number of grains in the ear and weight of grain from one ear) was analyzed by the sheaves, which were taken before harvesting from the sites of 1 m^2.

The structure of the crop was analyzed by the sheaves, which were taken before harvesting from the plot of of 1 m^2 in two non-adjoining reiteration. The average number of ears in the ear was determined by counting the number of ears per 25 spikes. The average mass of grain from one ear was calculated by dividing the weight of the grain of the spikes sample per the number of productive stems. The yield was determined by the method of continuous harvesting of each registration area (Sampo - 130 combine harvester).

The statistical analysis (repetition was three times during 5 years of growing grain) of the research were processed using the method of multivariate disperse analysis.

The obtained data were compared using analysis of variance (ANOVA). All statistical analyses were performed with Statistica 10, Agrostat New and Microsoft Office 2010.

RESULTS AND DISCUSSIONS

The crop structure is a quantitative expression of the result of the plant's vital activity, which determines the size of the yield and reflects the interaction of the plant and the environment at certain stages of its growth and development. Important components of the crop structure of spring barley are the coefficient of productive bruising, the length of the ear, the number of grains in the ear and the mass of 1000 grains (Girka et al., 2017).

The main factors which form the productive stalk are the genetic features of the variety, the nutrition of plants with nutrients and hydro-thermal conditions of the growing season. Tamm (2003) reported a relatively low genetic variability in the tillering among the European spring barley varieties, however, information on the differences in the relative proportion of individual tiller categories is not available.

The critical period for grain number determination is generally between the end of the tillering and the end of the stem elongation (Chmielewski and Köhn, 1999), so that a sufficient number of strong fellers at the beginning of this period are critical for achieving a high number of grains in the spike.

From the studied varieties of spring barley, on average, over the years of research and in terms of nutrition, somewhat higher density of productive stems, were formed by plants of the Aeneas variety as 379 pcs./m^2, while Adapt and Stalker plants were slightly smaller as 349 and 361 pcs./m^2 (Table 1). It should be noted a more pronounced response to the optimization of plantnutrition of the Aeneas variety, with this indicator varied from 341 to 401 pcs./m^2.

The largest number of productive stems in the studied spring barley varieties was formed on the background of mineral fertilizers in a dose of $N_{30}P_{30}$ under pre-sowing cultivation and conducting of extra-root crops fertilization in the main phases of growth and development of plants with the preparations Organik D2 and Escort-bio.

Table 1. The number of common and productive stems in plants of spring barley varieties depending on the optimization of plant nutrition (average for 2013-2017 years), pcs./m^2

Plant nutrition (factor B)	Variety (factor A)					
	Adapt		Stalker		Aeneas	
	total number of stems, pcs./m^2	number of productive stems, pcs./m^2	total number of stems, pcs./m^2	number of productive stems, pcs./m^2	total number of stems, pcs./m^2	number of productive stems, pcs./m^2
Control (without fertilizers)	364	315	383	324	411	341
$N_{30}P_{30}$ (background)	389	335	406	350	450	371
Background + Urea K1	402	348	421	361	499	379
Background + Urea K2	410	353	431	366	509	382
Background + Escort-bio	442	369	475	381	558	401
Background + Urea K1 + Urea K2	424	358	446	370	533	386
Background + Organic D2	435	363	463	372	543	391
$LSD_{0.5}$ 2013: factor A	13.0	6.9				
factor B	8.1	11.0				
2014: factor A	11.0	15.0				
factor B	9.0	7.0				
2015: factor A	12.0	5.0				
factor B	8.0	8.0				
2016: factor A	2.0	8.0				
factor B	8.0	7.0				
2017: factor A	6.0	4.0				
factor B	6.0	6.0				

According to the LSD test the difference between the studied variants of the experiment significant ($P > 0.05$).

Thus, in these variants of plant nutrition of the Aeneas variety, 391 and 401 pcs./m^2 of productive stems were formed, respectively, and Adapt and Stalker varieties were respectively 363-369 and 372-381 pcs./m^2, which exceeded the control respectively by 12.8-17.6; 13.2-14.6 and 12.9-15.0%.

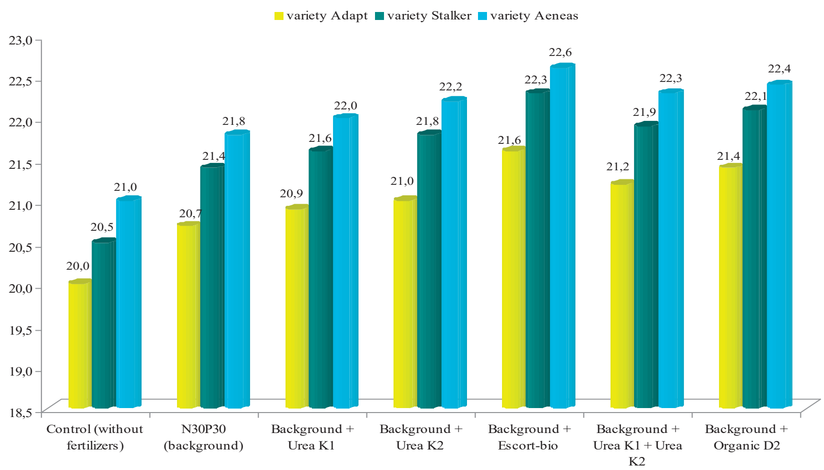
Somewhat lower density of productive stalk was formed by co-treatment of spring barley crops with substances Urea K1 and Urea K2 in the background of mineral fertilizers. Thus, on average, over the years of research, 1 m^2 at the same time there were 358-386 productive stems in terms of varieties, which exceeded the indices of variants without fertilization by 12.0-13.2%.

It should be noted that the application of mineral fertilizers in the moderate recommended dose of $N_{30}P_{30}$ contributes to the increasing of the specified indicator of the cropstructure of spring barley compared with the control of 6.0-8.1% depending on the variety. But compared with the variants of foliar fertilization (Urea K1, Urea K2, Organic D2, Escort-bio), the number of productive stems by fertilizing in dose of $N_{30}P_{30}$ was less than 3.7-9.2% for the cultivation of the Adapt variety, 3.1 to 8.1% for the cultivation of the Stalker variety and 2.1 to 7.5% for the Aeneas variety.

The dispersion analysis showed that the studied factors affect on the total number of stems per the spring barley plant. Thus, on average, over the years of research, the highest impact on this indicator had variety (factor A) - the share of the impact was 53.2%. The share of the impact of factor B was 42.4%. The interaction of the studied factors was 4.2%.

In the distribution of the impact of the studied factors on the number of productive stems, it was observedanother dependence. So, the factor of plant nutrition (factor B) provided 65.7%, the factor of variety (factor A) provided 32.7%, the interaction of both factors (factor A and B) provided 0.4%.

One of the most important elements which characterize the productivity of a spike of spring barley is the number of grains in the ear. Studies by Del Moral et al. (2003) shows that the stability of yield of spring barley varieties in various conditions is closely related to the number of grains in the ear. It provides the formation of the greater number of grains per area unit with the availability of the lesser number of ears. But the quantity of ears can decrease. And, conversely, according to Jockovic et al. (2014) the number of grains in the ear can not significantly affect the grain yield. Our research established that the specified element of the productivity of spring barley depended on the variety and plant nutrition variant (Figure 1).



$LSD_{0.5}$ (pcs.): Factor A (variety): 2013 - 2, 2014 - 4, 2015 - 8, 2016 - 7, 2017 – 2

Factor B – plant nutrition: 2013 - 1, 2014 - 2, 2015 - 2, 2016 - 4, 2017 - 1

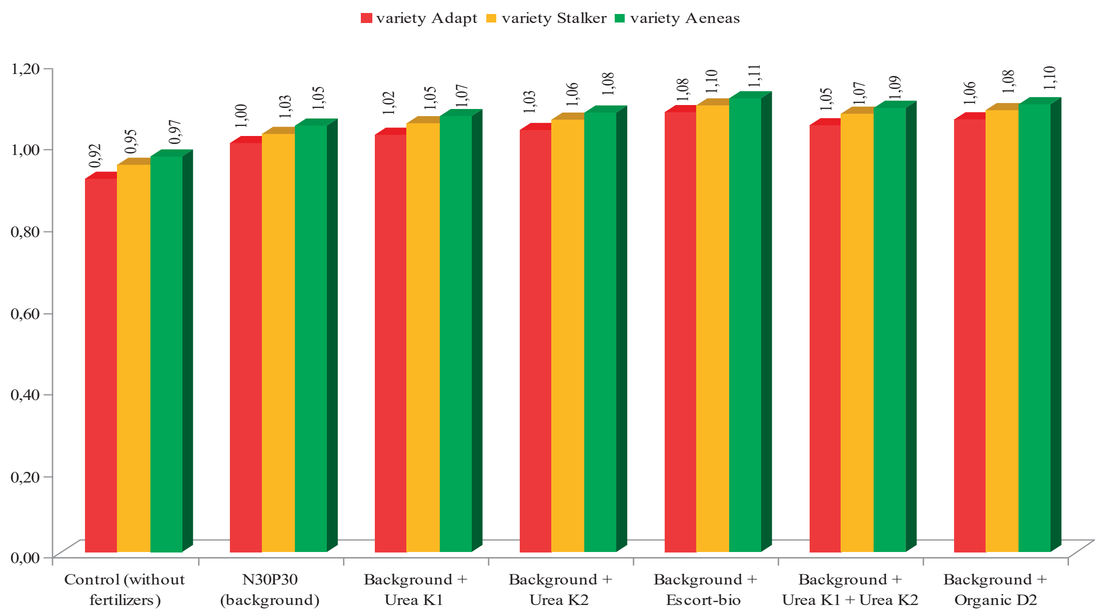
According to the LSD test the difference between the studied variants of the experiment significant ($P > 0.05$).

Figure 1. Number of grains in the ear of spring barley varieties depending on the optimization of plant nutrition, pcs. (average for 2013-2017)

Thus, on average, over the years of research, plant nutrtion variants have significantly influenced on the number of grains in the ear of the studied varieties of spring barley. So on the control (without fertilizers) the ear of the Adapt varieties plants numbered 20.0 grains, the ear of the Stalker variety numbered 20.5 pcs. and the ear of the Aeneas variety numbered 21.0 pcs., then the pre-planting of only mineral fertilizers provided the increasing of this index in terms of the studied varieties by 3.5-4.4%, and for conducting in the background of fertilizers of extra-root nutrition by 4.5-8.0% for the cultivation of the Adapt variety, by 5.4 to 8.8% for the Stalker variety, and by 4.8 to 7.6% for Aeneas variety.

A somewhat larger number of grains in the ear in all years of the research were formed by plants of the Aeneas variety. So, on average, over the years of research on the plant nutrition factor, they formed 22.0 pcs., which exceeded other varieties by 0.3-1.0 pcs. or 1.4-4.8%. The application of Organic D2 and Escort-bio contributed to an increase in the number of grains in the ear from 6.3 to 7.6% for the Aeneas variety.

We found that on average over the years of research, varieties and variants of plant nutrition affected on the mass of grain from one ear (Figure 2).



$LSD_{0.5}$ (pcs.): Factor A (variety): 2013 – 0.010, 2014 – 0.017, 2015 – 0.023, 2016 – 0.036, 2017 – 0.002
Factor B – plant nutrition: 2013 – 0.008, 2014 – 0.0013, 2015 – 0.019, 2016 – 0.032, 2017 – 0.003
According to the LSD test the difference between the studied variants of the experiment is not significant (P> 0.05).

Figure 2. Weight of grain from one ear of spring barley, depending on the variety and the background of nutrition, g (average for 2013-2017 years)

So, for the application of the background recommended dose of mineral fertilizers for spring barley in a variety of varieties Adapt the weight of grain from the ear in comparison with unchecked control increased by 9.4%, the weight of grain from the earof the Stalker variety increased by 8.0%, and weight of grain from the earof the Aeneas variety increased by 7.9%. Conducting of extra-root nutrition on the background of mineral fertilizers increased the specified index of crop yield structure, respectively, by 11.9-17.7; 10.6-15.5 and 10.2-14.7% for control.

Similarly, the studied factors were also reflected in the levels of grain yield of spring barley varieties (Table 2).

The given data testified that plant nutrition significantly influenced on the productivity of spring barley varieties. According to the results of the dispersion analysis, it was found that on the average over the years of research, the share of the impact of factor B (plant nutrition) was 74.5%. The factor A (variety) had a weaker effect on the crop yields as the share of impact was 23.9%. The interaction of factors A and B was 0.3%.

It was established by the research that application of Urea K1 and Urea K2 for foliar fertilization of plants increased the grain yield of spring barley. Thus, on average, over the years of research and by factor variety, it were formed 3.21 and 3.26 t ha^{-1} grains, which exceeded the control by 0.55-0.60 t ha^{-1} or by 20.7-22.6%, $N_{30}P_{30}$ (background) - by 0.15-0.20 t ha^{-1} or by 4.7-6.1%.

But compared to the use of Organic D2 and Escort-Bio, the yield of barley was somewhat lower by 3.3-4.7 and 4.4-5.9%. The co-administration of these drugs provided the grain yield of spring barley at almost the same level as 3.33 t ha^{-1}.

Yields are significantly dependent on the characteristics of the variety. At the present time, the spring barley and winter wheat variety acts as an independent factor in increasing of grain yield (Panfilova et al., 2018; Panfilova & Mohylnytska, 2019).

Table 2. Yield of spring barley depending on varietal characteristics and optimization of plant nutrition, t ha^{-1}

Variety (factor A)	Plant nutrition (factor B)	Years					Average for 2013-2017 yrs
		2013	2014	2015	2016	2017	
Adapt	Control (without fertilizers)	2.25	2.61	2.55	2.86	2.52	2.56
	$N_{30}P_{30}$ (background)	2.51	2.96	2.90	3.28	2.89	2.91
	Background + Urea K1	2.69	3.10	3.08	3.46	2.93	3.05
	Background + Urea K2	2.71	3.14	3.10	3.59	3.00	3.11
	Background + Escort-bio	2.83	3.27	3.21	3.75	3.20	3.25
	Background + Urea K1 + Urea K2	2.74	3.21	3.14	3.65	3.12	3.17
	Background + Organic D2	2.79	3.24	3.18	3.71	3.18	3.22
Stalker	Control (without fertilizers)	2.34	2.69	2.62	2.88	2.64	2.63
	$N_{30}P_{30}$ (background)	2.66	3.09	3.01	3.30	3.06	3.02
	Background + Urea K1	2.79	3.20	3.18	3.65	3.15	3.19
	Background + Urea K2	2.81	3.23	3.20	3.70	3.22	3.23
	Background + Escort-bio	2.95	3.36	3.31	3.84	3.39	3.37
	Background + Urea K1 + Urea K2	2.86	3.29	3.26	3.76	3.30	3.29
	Background + Organic D2	2.91	3.32	3.29	3.80	3.35	3.33
Aeneas	Control (without fertilizers)	2.36	2.80	2.79	3.18	2.89	2.80
	$N_{30}P_{30}$ (background)	2.73	3.21	3.22	3.75	3.31	3.24
	Background + Urea K1	2.94	3.40	3.29	3.94	3.34	3.38
	Background + Urea K2	2.99	3.48	3.35	4.01	3.36	3.44
	Background + Escort-bio	3.12	3.58	3.52	4.30	3.51	3.61
	Background + Urea K1 + Urea K2	3.06	3.51	3.42	4.22	3.41	3.52
	Background + Organic D2	3.08	3.56	3.47	4.25	3.45	3.56
$LSD_{0.5}$	factor A	0.08	0.10	0.09	0.08	0.11	
	factor B	0.11	0.13	0.14	0.10	0.13	

According to the LSD test the difference between the studied variants of the experiment significant (P > 0.05).

According to Ayranci et al. (2014) and Ahmadi et al. (2016), the grain yield is more dependent on the environment during the growing season than on the genotype effect.

The application of a moderate dose ($N_{30}P_{30}$) of mineral fertilizers contributed to the slight increasing in the grain yield of winter wheat in all years of research irrespective of the variety (Panfilova et al., 2018). At the same time, studies (Ammanullah, 2014; Sedlar et al., 2017) showed that moderate doses of nitrogen fertilizers (50-100 kg ha^{-1}) had a slight effect on the grain yield. At the same time, the grain yield of corn increased with an increase in the dose of N to 150 kg ha^{-1}. In our studies, the application of a moderate dose ($N_{30}P_{30}$) of mineral fertilizers contributed to the slight increasing in the grain yield of spring barley in all years of research irrespective of the variety - by 0.35-0.44 t ha^{-1}.

According to the results of our research, it was established that, in addition to weather conditions and plant nutrition options, the variety played an important role in the formation of spring barley yield. Thus, on average, over the years of research on nutrition, the highest grain yield was formed by Aeneas plants as 3.36 t ha^{-1}, which exceeded its level in the Stalker variety by 0.21 t ha^{-1}or 6.3%, and the Adapt variety by 0.32 t ha^{-1} or 9.5% respectively. According to the LSD test the difference between the studied variants of the experiment significant ($P > 0.05$).

On average, over the years of research, it was proved that the grain yield of spring barley depended on the studied characteristics of the yield structure. Thus, according to the results of the correlation analysis, it was established the strong correlation between the yield and the number of productive stems of the plant (R^2 = 0.979), the yield and the number of grains in the ear (R^2 = 0.974), the yield and the weight of grains from the ear (R^2 = 0.949).

CONCLUSIONS

In the conditions of southern Ukraine, the application of mineral fertilizers at a dose of $N_{30}P_{30}$ under pre-sowing cultivation and the implementation of foliar nutrtionof crops at the beginning of the phase of spring barley stooling and earing with the preparations Urea K1, Urea K2, Escort-bio and Organic D2 provides the best conditions for the growth and development of plants and, as a consequence, the formation of more optimal indicators of the yield structure and grain yield. Thus, according to these plant nutrtion options, on the average over the years of research, the number of productive stems in plants of the Aeneas variety was 386-401 pcs./m^2, and it was in the Adapt and Stalker varieties, 358-369 and 370-381 pcs./m^2. In this regard, irrespective of the year of cultivation, the highest grain yield of spring barley was formed by the application of mineral fertilizers in a dose of $N_{30}P_{30}$ and nutrition of plants with the preparation Escort-bio. On average, over the years of research, in this version of the plant nutrtion, the highest level of grain productivity among the studied varieties was provided by the variety Aeneas as 3.61 t ha^{-1}. When fertilizing with other treatments, the grain yield of the Aenei variety was not significantly less - 3.52-3.56 t ha^{-1}.

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BIOCHEMICAL CHANGES UNDER ARTIFICIAL LED LIGHTING IN SOME *Lactuca sativa* L. VARIETIES

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Abstract

Relationship between dietary habits and disease risk was demonstrated by numerous epidemiological studies which led to the conclusion that food has a direct impact on health. On the other hand, recent researches established some correlations between the consumption of fresh fruits and vegetables and their health benefits. Lettuce (Lactuca sativa L.) is an important dietary leafy vegetable mainly consumed fresh or in salad mixes for its good taste, but also because it is perceived as being amongst healthy food since a number of lettuce varieties have been investigated and reported to contain some compounds with antioxidant activities.
Lettuce cultivation has been greatly expanded in recent times, so that researchers have been looking for solutions for growing it in confined spaces using artificial LED lighting (light emitting diodes). The objective of this research was to investigate the effect of LED lighting on biochemical characteristics of some lettuce varieties in order to establish the best light treatment during cultivation for enhancing the plants growth, yield and nutritional value. Different combinations of LED wavelengths against conventional light sources were investigated and the researches performed demonstrated that LEDs variably influenced growth characteristics and increased biochemical compounds content in the analyzed lettuce varieties.

Key words*: chlorophylls, LED lighting, lettuce, sugars, total phenolics.*

INTRODUCTION

In recent years relationship between dietary habits and disease risk was demonstrated by numerous epidemiological studies so that many researchers have shown an increased interest in vegetables and fruits, not only due to their nutritional value, but also to their content in active ingredients capable of providing health benefits (Kris-Etherton et al., 2002; Gundgaard et al., 2003; Balan et al., 2016; Pinela et al., 2016).
Lettuce (*Lactuca sativa* L.) is an important dietary leafy vegetable mainly consumed fresh or in salad mixes for its good taste, but also because it is perceived as being amongst healthy food. It is a good source of fibre, iron, folate, vitamin C and various other health-beneficial bioactive compounds. Different studies reported anti-inflammatory, cholesterol-lowering and anti-diabetic activities attributed to the bioactive compounds in lettuce (Hedges & Lister, 2005; Kim et al., 2016).
Lettuce cultivation has been greatly expanded in recent times, but farmers are faced with price increases to provide additional lighting in greenhouses, especially when the light is low, because using fluorescent or incandescent lamps causes high power consumption. Although these light sources induce an increase of photosynthesis levels, they are not as energetically efficient as desired. Moreover, spectral quality influences plant growth and development, but the previously mentioned light sources do not offer the option of spectral manipulation (Bantis et al., 2016). LED (light emitting diodes) technology presents numerous advantages such as long life, small volume, low heat emission, adjustable light intensity, high energy-conversion efficiency and wavelength specificity (Morrow, 2008; Massa et al., 2008).
Along with LED energy-saving and functionality, their safety for environment should be mentioned (Olle & Viršile, 2013) in the context in which the public interest in

environmental protectionis a priority of any activity nowadays (Moteva, 2016).
However, one of the most highlighted LEDs advantages is the possibility to select only specific light wavelengths and therefore to optimize the spectral quality for various plants and different physiological processes such as growth, flowering, photosynthetic efficiency (Olle & Viršile, 2013). Red and blue light are the basal component in lighting spectra for green vegetables; exclusive red light is sufficient for normal plant growth and photosynthesis (Goins et al., 2001) while far red light is important for photomorphogenetic processes and also promotes plant growth (Olle & Virsile, 2013). Blue light also causes positive effects on green vegetable morphology, growth and photosynthesis activating cryptochrome system and matching chlorophyll and carotenoids absorption spectra (Yanagi et al., 1996). Using red LED light to power photosynthesis has been widely accepted mainly because there was indicated that red wavelengths are efficiently absorbed by plant pigments (Sager & McFarlane, 1997).
The other main wavelength included in early studies has been in the blue region of the visible spectrum. The amount of blue light required and optimal for different species were investigated. Blue light has a variety of important photomorphogenic roles in plants, such as phototropism (Blaauw & Blaauw-Jansen, 1970) and stomatal opening (Schwartz & Zeiger, 1984).
Knowledge on the response of vegetable plants to light quality allows growers to influence one or the other side of growth and development processes.
As light emitting diodes represent a promising technology for the greenhouse industry some plant species such as lettuce (Kim et al., 2004; Li & Kubota, 2009; Ouzounis et al., 2015), sweet basil (Fraszczak et al., 2014), tarragon (Enache & Livadariu, 2016), cucumber and tomato seedlings (Brazaityte et al., 2009; Brazaityte et al., 2010) have been tested with good results. It seems that using LED lighting provide high productions, shortening the crop cycle, uniform plant development, good quality, low water consumption and finally leads to an overall reduction in crop production costs.
The objective of this research was to investigate the effect of LED lighting on biochemical characteristics of some lettuce varieties in order to establish the best light treatment during cultivation for enhancing the plants growth, yield and nutritional value. Biochemical determinations of the content in nutritive compounds (sugars, proteins) and antioxidants (vitamin C, total phenolics, chlorophylls, carotenoids) were performed on the fresh leaves. Thus, the study was also designed to improve knowledge concerning the nutritional value of some lettuce varieties and their potential to provide essential nutrients, which can improve health-related quality of life.

MATERIALS AND METHODS

The experiment was conducted in the NFT (Nutrient Film Technologies) cultivation department from the experimental greenhouse of the Hortinvest Research Centre - USAMV Bucharest in April-May period.
As biological material two lettuce varieties were used. Lettuces are grouped in different commercial types according to some agronomic characteristics, like the capacity for sprouting, the consistency of the leaves or the adaptation to a given season.

Figure 1. Experimental variants ‘Touareg F1’ (Control, LED 1, LED 2)

The researches included in the present paper were performed on green lettuce ‘Touareg F1’ (Figure 1) and on classic ‘LolloBionda’ (Figure

2) which is a green-leaved lettuce with strongly curled leaves. The lettuce plants were cultivated using different sources of light.
Observations and determinations were made at the end of the culture cycle, when the salad plants have achieved consumption characteristics.

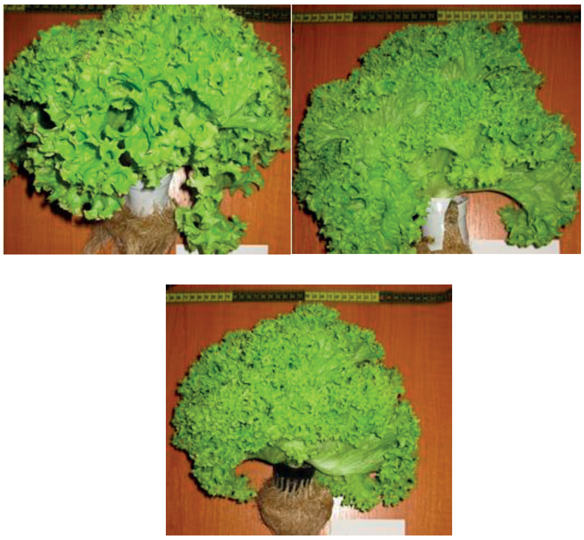

Figure 2. Experimental variants 'LolloBionda' (Control, LED 1, LED 2)

The experimental variants were (Figures 1 and 2):

- Control variant-lettuce plants cultivated under natural illumination, without supplemental lighting;
- LED 1 variant - lettuce plants cultivated under natural illumination + supplemental light provided by LED 1 installation (Figure 3);

Figure 3. LED 1 installation

- LED 2 variant - lettuce plants cultivated under natural illumination + supplemental light provided by LED 2 installation (Figure 4).

LED lighting panels were located at 1.2 m above the plants. Each panel, 80/60 cm in size, contained 180 LEDs. Additional lighting duration: 12 hours/day between 8-20 hours.

Figure 4. LED 2 installation

The spectral composition of the LED installation was:

- LED 1 installation- 70% red LEDs (640 nm) in combination with 30% blue LEDs (430 nm) (Figure 3);
- LED 2 installation- 30% red LEDs (640 nm) in combination with 70% blue LEDs (430 nm) (Figure 4).

Biochemical determinations of the content in nutritive compounds (sugars, proteins) and antioxidants (vitamin C, total phenolics, chlorophylls, carotenoids) were performed in triplicate using fresh leaves.
The extractions were conducted according to the protocol used for each determination.
Total soluble sugars were estimated according to the Somogyi-Nelson method (Iordachescu & Dumitru, 1988; Somogyi, 1952). Hydrolysis with hydrochloric acid was performed in order to transform non-reducing sugars in reducing ones which were heated with alkaline copper tartrate so that cuprous oxide was formed. The addition of arsenomolybdic acid determined the formation of molybdenum blue complex. The measurements of absorbance were made at 620 nm. The results were expressed in g% fresh weight.
Crude protein was determined after the digestion of the organic matter with sulfuric acid in the presence of a catalyst (Iordachescu & Dumitru, 1988; Kjeldahl, 1883). The content in total nitrogen was measured by volumetrical method. To obtain the crude protein content, the nitrogen content was multiplied by a conventional factor (6.25). The results were expressed in g% fresh weight.
Total phenolics concentration in the extract was performed spectrophotometrically according to the modified Folin-Ciocalteu assay (Singleton et al., 1999). The method is based on the colour reaction of the Folin-Ciocalteu reagent with the hydroxyl groups.

The intensity of the obtained blue colour was measured at 750 nm. The total phenolics amount was calculated from the calibration curve using gallic acid as a standard and the results were expressed as milligrams of gallic acid equivalents per 100 grams fresh weight of extract (mg GAE/100 g fresh weight).

Vitamin C content was evaluated by spectrophotometric method with a dye solution of 2.6-dichlorophenol indophenol (Artenie & Tanase, 1981). Extraction of ascorbic acid was made in 2% oxalic acid. Absorbance was measured at 500 nm. Results were expressed as mg/100 g fresh weight.

Assimilatory pigments content was determined after *chlorophyll* and *carotenoid pigments* were extracted in 80% acetone. Absorbance was measured at 663 nm, 647 nm and 480 nm. Total chlorophylls and total carotenoids have been calculated using the extinction coefficients and equations described by Schopfer (1989). The results were expressed in mg/100 g fresh weight.

Dry matter content was analysed by gravimetric method with oven drying: samples had been dried to constant mass at $(105 \pm 5)^{o}$C and the loss of weight is used to calculate the dry matter content of the sample.

Statistical analysis were made using One-way Analysis of Variance (ANOVA). The measurements were done in triplicate and all the data were expressed as mean ± S.D. Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSIONS

The most important factor influencing plant growth is light exposure. Light can condition the development of plant through intensity, duration and spectral composition. The light spectrum radiation is selectively absorbed by chlorophyll pigments, so light quality directly influences physiological and chemical processes in plants. Yanagi et al. (1996) reported that lettuce plants cultivated under red LEDs alone had more leaves and longer stems than plants grown under blue LEDs only. Yorio et al. (1998) showed that yield of lettuce crops grown under red LEDs alone was reduced compared to variant with blue fluorescence included to give the same final PPF (photosynthetic photon flux). Leaf morphology was abnormal for lettuce plants grown under red light alone with downward curling of leaf margins and spiral growth of the rosette, but inclusion of blue light at any level restored normal leaf morphology (Goins et al., 1998).

Analysis of nutritive compounds

The results obtained in the present study regarding the nutritive compounds (Table 1) showed that the analysed lettuce varieties registered similar values of the biochemical content of the control plants. Thus, similar values of *dry matter* content (4.35 g% in ‘Touareg’ variety, respectively 4.85 in ‘LolloBionda’ leaves) were registered in the green lettuce varieties exposed only to natural lighting, in accordance with results reported in previous study by Luta et al. (2020) (5.93 g% *dry matter* in lettuce cultivated also in greenhouse). However, *total soluble sugars* content was higher in the lettuce leaves of ‘Touareg’ variety (0.25 g% FW) compared to ‘LolloBionda’ leaves (0.12 g% FW). The amounts of *crude protein* determined in the salad lettuce (1.46 g% FW in ‘Touareg’, respectively 1.21 g% FW in ‘LolloBionda’) were comparable to the ones reported by other studies which showed that protein concentrations in green lettuce leaves were influenced by growing season in floating raft culture (13.2-16.5 mg/g FW in spring season and 10.2-15.2 mg/g FW in summer season) (Fallovo et al., 2009).

Instead, supplementing the light source with artificial LED enhances the accumulation of nutritive compounds in the studied varieties of lettuce, higher values being registered for both dry matter, sugars and protein content compared to the control plants.

LED 1 lighting leads to an increase of *sugars* content with 70% in ‘Touareg’ variety and with almost 80% in ‘LolloBionda compared to control variants (Table 1). Also, *proteins* content registered higher values in the lettuce plants cultivated under LED 1 exposure, the increase being of 14% in ‘Touareg’, respectively 26% in ‘LolloBionda’ variety.

LED 2 lighting determined changes in the chemical composition of lettuce leaves too, but the results are different. Thus, a lower accumulation of the *sugars* in the lettuce plants was registered under these conditions compared to the LED 1 variant (for 1.6 times lower in 'Touareg' variety, respectively for 1.2 times lower in 'LolloBionda' variety), but significant increased compare to control plants were noted (Table 1). Instead the *proteins* content registered in the LED 2 variants were similar to those measured for the control plants.

No significant differences between LED variants were noted regarding the *dry matter* content in both studied lettuce varieties.

Table 1. Variability of some nutritive compounds in the leaves of *Lactuca sativa* varieties

Lactuca sativa	Samples	Dry weight (g%)	Total soluble sugars (g% FW)	Crude protein (g% FW)
var. 'Touareg'	Control	4.35 ± 0.16^a	0.25 ± 0.01^a	1.46 ± 0.08^a
	LED 1	5.04 ± 0.20^b	0.83 ± 0.05^b	1.70 ± 0.09^b
	LED 2	5.56 ± 0.24^b	0.51 ± 0.03^c	1.43 ± 0.08^a
var. 'LolloBionda'	Control	4.85 ± 0.20^a	0.12 ± 0.01^a	1.21 ± 0.05^a
	LED 1	5.25 ± 0.19^b	0.58 ± 0.03^b	1.65 ± 0.07^b
	LED 2	5.12 ± 0.16^b	0.47 ± 0.02^c	1.20 ± 0.05^a

Note: Data expressed as mean values (n = 3) ± standard deviation; different superscript letters within the same column and variety indicate significant differences (P < 0.05).

These results demonstrate that LEDs variably influenced biochemical characteristics in the lettuce plants pointing out that LED light with predominantly red component enhances photosynthesis process therefore the accumulation of metabolites. Red light usually is the basal component in lighting spectra and using red light alone is sufficient for a good plant growth and development (Olle & Viršile, 2013). However, different wavelengths of red light might have different effects on plants. Goins et al. (2001) showed that biomass yield of the lettuce plants increased when the wavelength of red LED increased from 660 to 690 nm.

At the same time, positive effects of blue light were also demonstrated on green vegetable morphology, growth and photosynthesis (Yanagi et al., 1996).

Analysis of antioxidant compounds

For the control plants the lowest amount of *vitamin C* was recorded in the 'Touareg' lettuce leaves (3.43 mg/100 g FW) compared to 'LolloBionda' (7.89 mg/100 g FW) (Figure 5), in accordance with data reported by Aćamović-Djoković et al. (2011), which found high variability of vitamin C content in some lettuce varieties (9.60 mg/100 g FW for Levistro, but only 3.50 mg/100 g FW in the Murai variety).

LED lighting determined an increase of vitamin C accumulation in the leaves for both lettuce varieties, significant differences being registered with LED 1 light combination: 2.76 times higher in 'Touareg' variety and 1.32 times higher in 'LolloBionda' variety compared with control plants.

Exposure at LED 2 combination leads also to higher amounts of vitamin C compared to control plants for 'Touareg' variety (for 2.26 times higher), while no significant difference was registered in 'LolloBionda' leaves (Figure 5).

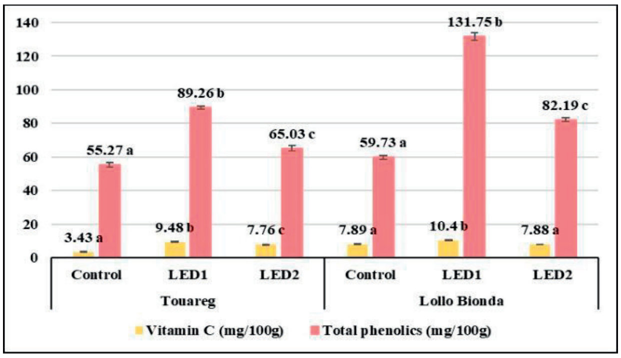

Figure 5. Variability of some antioxidants in the leaves of *Lactuca sativa* varieties

Note: Data expressed as mean values (n = 3) ± standard deviation; error bars represent the standard deviation; different superscript letters within the same variety indicate significant differences (P < 0.05).

Total phenolics contents determined in the analysed lettuce variety (Figure 5) reached similar values (55.27 mg GAE/100 g FW in 'Touareg', respectively 59.73 mg GAE/100 g FW in 'LolloBionda' variety) that correspond to the values determined by other authors.

For example, Luta et al. (2020) reported 55.36 mg GAE/100 g FW in *Lactuca sativa* grown in greenhouse, while Llorach et al. (2008)

determined values between 18.2-125.5 mg/100 g FW polyphenols studying several varieties of lettuce.

Analysis performed on lettuce varieties cultivated under LED lighting showed higher amounts of phenolics compared to the control variants. Thus, a significant increase was noted in the lettuce plants illuminated with LED 1 combination: almost 55% in 'LolloBionda' variety, respectively 38% in 'Touareg' variety. Also LED 2 lighting stimulated the phenolics accumulation, but the increase was lower in relation to the values determined in the control plants: only 15% in 'Touareg' variety and 27% in 'LolloBionda' leaves.

Although these results revealed a lower influence of LED 2 combination (with blue light added in a larger proportion) on biochemical compound accumulation, different authors reported that supplemental blue LED light leads to increased content of some antioxidants in green vegetables: polyphenols (Johkan et al., 2010), vitamin C (Li et al., 2012), carotenoids and anthocyanins (Li and Kubota, 2009).

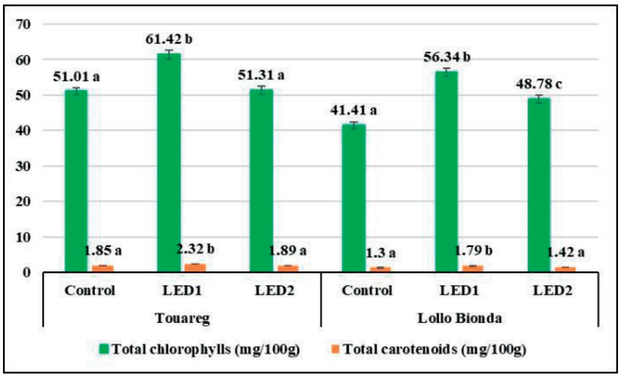

Figure 6. Variability of assimilatory pigments in the leaves of *Lactuca sativa* varieties

Note: Data expressed as mean values (n = 3) ± standard deviation; error bars represent the standard deviation; different superscript letters within the same variety indicate significant differences ($P < 0.05$).

Assimilatory pigments, meaning total chlorophylls and total carotenoids in the lettuce varieties were analysed too (Figure 6). The antioxidant potential of carotenoids and its role in preventing some cancer are known, however for chlorophyll also were investigated anti-cancer activity (Hedges & Lister, 2005).

Analysis of assimilatory pigments content in the control lettuce plants revealed higher content of total chlorophylls (51.01 mg/100 g FW) and carotenoids (1.85 mg/100 g FW) in 'Touareg' variety compared to 'LolloBionda' variety (41.41 mg/100 g FW chlorophyll and 1.3 mg/100 g FW carotenoids) (Figure 6).

Cultivation of the lettuce plants under LED 1 lighting stimulated chlorophyll accumulation whose amounts registered increasing in proportion of 17% in 'Touareg' variety and 26.5% in the 'LolloBionda' leaves compared to the control plants.

Also, illumination with LED 2 combination has resulted in higher amounts of total chlorophyll in 'LolloBionda' variety (with 15%) than in control plants, while in 'Touareg' no significant effect was registered. However, there are studies reporting that blue LEDs (440-476 nm), used alone or in combination with red LEDs, caused higher chlorophyll ratio and stimulated biomass accumulation in Chinese cabbage plants (Mizuno et al., 2011; Li et al., 2012) and in lettuce (Johkan et al., 2010).

The evolution of carotenoids under LED lighting is similar: an increase of 20% in the leaves of 'Touareg' and of 27% at 'LolloBionda' variety compare to the control plants was noted when LED 1 combination was used. LED 2 lighting produced no significant differences regarding the carotenoids amount in both studied lettuce varieties.

CONCLUSIONS

This study shows that the lettuce varieties analysed are good sources of bioactive compounds, such as vitamin C and carotenes and showed promising antioxidant properties, which are related to their high levels of phenolics. Their nutritional potential could make them attractive, given the increasing awareness of consumers that food is important for improving well-being and quality of life.

The researches performed revealed that supplementing the natural light source with artificial LED enhances the accumulation of nutritive compounds in the studied varieties of lettuce, higher values being registered for both dry matter, sugars and protein content compared to the control plants.

Additional LED lighting also resulted in higher amounts of antioxidants like vitamin C, phenolics, carotenoids and chlorophylls in the leaves of the lettuce varieties analysed compared to the plants cultivated without supplemental light.

The highest effects of increasing the bioactive compounds accumulation in the lettuce leaves was noted for LED 1 lighting installation, with predominantly red light in composition.

These preliminary experiments demonstrate that spectral quality of light can influence plants development showing a favourable effect of LED lighting which offers the possibility of spectral manipulation therefore the LED technology can be promising for greenhouse horticulture.

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DEVELOPMENT OF AN EFFECTIVE TECHNIQUE FOR *IN VITRO* *Agrobacterium* - MEDIATED GENETIC TRANSFORMATION OF WINTER RAPE *Brassica napus* L.

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Abstract

The aim of the research was to improve the genetic transformation and adaptation conditions of commercial winter rapeseed line Brassica napus L. with subsequent production of transgenic seeds.It was optimized the technique of Agrobacterium-mediated genetic transformation of winter rapeseed Bn1 line (Brassica napus L.) using hypocotyls of 6-day-old seedlings as explants. GUS activity histochemical analysis showed a positive reaction in calli cultures and regenerant plant leaves obtained after Agrobacterium tumefaciens GV3101 transformation, which carried pCB203 plasmid with gus and bar genes. The optimal transformation conditions were determined as follows: the bacterial suspension optical density at a 600 nm wavelength - OD = 0.5, 10 min of inoculation and 48 h of co-cultivation. Stable integration of marker genes was confirmed by both histochemical and PCR analysis.

***Key words**: Brassica napus, winter rapeseed, genetic transformation, bar gene, gus gene.*

INTRODUCTION

In many countries of the world, rapeseed is primarily cultivated as an oilseed. In the United States, canola's oil has an official safe status for human consumption since 1985. The improvement in the rapeseed oil quality has caused a sharp increase in demand for it throughout the world. Oilseed rape varieties – winter (*Brassica napus* var. *oleifera biennis*) and spring (*Brassica napus* var. *oleifera annua*) do not differ morphologically (U S Pharmacopeia, 1989). However, among the oilseed *Brassicaceae* family winter rape takes first place in oil content, which in its seeds contain 48-52%, 16-29% of proteins, 6.7% of fats and 17% carbohydrates. Rapeseed oil consumes in kind, and it is the best material for the production of sandwich butter, margarine, mayonnaise, dressings and confectionery. Rapeseed oil also produces plastics, paints, varnishes.

In order to improve the genetic characteristics of rapeseed, protocols have been developed for its *Agrobacterium*-mediated transformation since the late 1980s. (Fry et al., 1987; De Block et al., 1989; Moloney et al., 1989). Genetic transformation using agrobacteria has several advantages and remains one of the most common methods for modifying the plant genome. The efficiency of genetic transformation depends on many factors, including the genotype of the original plant, its susceptibility to transformation, explants type, transformation and cultivation conditions. The ability to regenerate plants from transformed cells is also essential for successful transformation (Bhalla & Singh, 2008).

Although some commercial rapeseed lines have already been successfully transformed (Bhalla & Singh, 2008; Mashayekhi et al., 2008; Rahnama & Sheykhhasan, 2016; Bates et al., 2017), to maximize the release of transgenic plants the *Agrobacterium*-mediated genetic transformation protocol should be optimized for each individual genotype. The urgency of the work is that the transformation conditions for the Ukrainian breeding winter rapeseed commercial line should be optimize. This protocol allows obtaining the first transgenic plants in 3-4 months after the beginning of the experiment, and the seeds in 10 months. Thus, this technique can be use in further studies to create biotechnological rapeseed plants with improved genetic characteristics.

MATERIALS AND METHODS

Plant material and *in vitro* culture

Seeds of *Bn1* winter rape line were kindly provided by Ltd "Ukrainian Scientific Institute of Plant Breeding" (VNIS). Seeds were surface sterilized according to the following method: 70% ethyl alcohol treatment for 5 min, soaking in 1.5% sodium hypochlorite solution for 20-30 min followed by washing in sterile distilled water three times for 5 min. After sterilization, the seeds were planted on a hormone-free nutrient medium MS (Murashige & Skoog, 1962), supplemented with 400 mg/l of antibiotic Ceftriaxone (MSG) and cultivated for 24 h in the dark at 24°C. Further, the dishes were cultured for another 5 days in the culture room at 16 h photoperiod and 24°C.

Callus induction and regeneration

To induce callus formation and shoot regeneration a modified technique (Rahnama & Sheykhhasan, 2016) was used.

3-day old calli cultures obtained from hypocotyl segments (0.5-1 cm) of 6-day old rapeseed seedlings on MS nutrient medium supplemented with 1 mg/l 2.4-Dichlorophenoxyacetic acid (2.4-D) (MSC) were used as explants for genetic transformation.

The organogenesis induction in the transformed tissues was performed on MS nutrient medium supplemented with 4 mg/l 6-Benzylaminopurine (BAP), 2 mg/l N6-(Δ2-Isopentenyl) adenine (2-iP), 5 mg/l $AgNO_3$ and 5 mg/l DL-Phosphinothricin (ppt) as a selective agent (MSO). After 2-3 weeks of cultivation, when the adventitious buds were formed, the explants were transferred to regeneration MS medium containing 3 mg/l BAP, 2 mg/L 2-iP and 8 mg/l ppt (MSR). The shoots elongation was carried out on nutrient MS medium with half macro- and micro-salts content and supplemented with 0.1 mg/l BAP and 3 mg/l ppt (MSE). Rooting of plants was performed on a hormone-free MS medium with half macro- and micro-salts content and supplemented with 3 mg/l ppt (MSA). All media were supplemented with 400 mg/l Ceftriaxone (Ct); the composition of all media is shown in Table 1.

Adaptation and vernalization of plants

The regenerated plants were planted in a peat mixture and grown under greenhouse conditions (16 h photoperiod, 24°C) for 6-8 weeks. For vernalization, the plants were placed in a climate chamber with a temperature 4ºC and an 8 h photoperiod for 8 weeks. After the vernalization, the plants were grown under greenhouse conditions (24°C, 16 h photoperiod); the bud formation was observed after 4-5 weeks. To obtain seeds, all peduncles were covered with plastic insulators and self-pollinated.

Bacterial strains and vectors

Agrobacteria *Agrobacterium tumefaciens,* strain GV3101, containing plasmid pCB203, was used for *Agrobacterium*-mediated gene transfer. Bacterial strain GV3101 (Holsters et al., 1980) was obtained from the Genetic Engineering department Bank of Bacterial strains and Plasmids of the Institute of Cell Biology and Genetic Engineering (Kyiv, Ukraine). The construct contains a β-glucuronidase (*gus*) reporter gene and a selective phosphinothricin acetyltransferase (*bar*) gene that provides plant cells resistance to the Basta® herbicide (the active substance - L-phosphinothricin). The product of the *bar* gene activity is phosphinothricin acetyltransferase, an enzyme that neutralizes phosphinotricin, providing growth and rooting of transgenic plants on a selective medium. Polyubiquitin-1 (Ubi1) promoter and maize introns were used in the vector to achieve high expression levels of these genes (Christensen & Quail, 1996) (Figure 1).

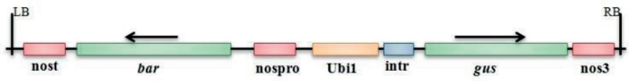

Figure 1. Schematic representation of the pCB203 vector T-DNA

Genetic transformation

A. tumefaciens GV3101 was grown overnight at 28ºC for 18 hours in LB medium (Bertani, 1951), which contained following antibiotics: Rifampicin (Rf) - 50 mg/l, Ampicillin (Amp) -

100 mg/l, Gentamicin (Gm) - 25 mg/l. The bacterial cells were centrifuged and resuspended in the inoculation medium MSI (Table 1). So that the optical density of the bacteria suspension was 0.2, 0.5 or 1.0, at a wavelength of light 600 nm. The inoculation of the pre-cultured hypocotyl explants was performed for 10, 30 or 60 minutes, followed by drying on sterile filter paper. Then the explants were placed on MSC medium (Table 1) and co-cultured in the dark at 24°C. After 48 hours, they were transplanted to MSC medium supplemented with 400 mg/l antibiotic Ceftriaxone to eliminate bacterial cells and cultured at the same conditions for another 10-12 days. Next, the explants were transplanted onto MSO medium (Table 1) supplemented with 5 mg/l ppt for selection of transformed cells followed with increasing of the amount of herbicide in the MSR medium (Table 1) up to 8 mg/l. All media at this stage contained 400 mg/l Ct. Every two weeks explants were transferred onto the fresh medium.

Total DNA extraction

The total DNA was extracted by CTAB method (Sambrook et al., 1989) from leaf material of regenerated canola plants, *B. napus* L., obtained after *in vitro* transformation using *A. tumefaciens* pCB203 genetic construct.

Polymerase chain reaction

The reaction mixtures included: specific primers, 2 µl of PCR buffer 10xDreamTaq™ GreenBuffer (Thermo Scientific) 0.2 mM of each deoxyribonucleoside triphosphate (Thermo Scientific), 2 units polymerase DreamTaq™ DNA Polymerase (Thermo Scientific), 100 ng of total DNA. The reaction mixture was adjusted to a final volume of 20 µl with deionized water Milli-Q.

GUS forward primer: 5'-ATG-GGT-CAG-TCC-CTT-ATG-TTA-3'

GUS reverse primer: 5'-ATA-AAG-ACT-TCG-CGC-TGA-T-3'

The expected band size - 239 bp.

The reactions were performed using the following profiles: initial denaturation 5 min at 95°C, 40 cycles - denaturation 40 sec at 95°C, annealing 40 sec at 50°C, elongation 45 sec at 72°C, final elongation 7 min at 72°C.

Histochemical GUS expression

To analyse the *gus* gene expression in transformed plants, histochemical analysis of GUS activity was performed using method (Jefferson, 1987). The organ and callus fragments were incubated overnight with 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) solution followed by washing in 70% ethyl alcohol.

Table 1. The composition of the nutrient media used in the study

Designation of nutrient medium	Basic salt composition	Sucrose, g/l	Agar-agar, g/l	pH	Growth regulators, mg/L			Other components		
					2.4-D, mg/l	BAP, mg/l	2-iP, mg/l	$AgNO_3$, mg/l	Ct, mg/l	ppt, mg/l
MSG	MS	20	7	5.7-5.8	-	-	-	-	400	-
MSC	MS	30	7	5.7-5.8	1	-	-	-	-	-
MSI	1/2 MS	20	-	5.2	-	-	-	5	-	-
MSO	MS	30	7	5.7-5.8	-	4	2	5	400	5
MSR	MS	30	7	5.7-5.8	-	3	2	-	400	8
MSE	1/2 MS	20	8	5.7-5.8	-	0.1	-	-	400	3
MSA	1/2 MS	20	8	5.7-5.8	-	-	-	-	400	3

Statistical data processing

The experiments were conducted in three replicates. The regeneration frequency of transformed tissues was calculated as the ratio of the explants number that formed organs on a selective medium to the total number of explants used in the experiment. The transformation frequency was calculated as the ratio of PCR-positive plants to the original number of explants. The results were statistically processed using Microsoft Office Excel.

RESULTS AND DISCUSSIONS

The aim of the research was to improve the *Agrobacterium*-mediated genetic transformation conditions (the duration of inoculation with the agrobacterial suspension, the optical density of the suspension and the composition of the inoculation and regeneration media) of commercial winter rapeseed Ukrainian breeding line in order to develop a cost-effective and efficient transformation technique.

Callus induction and regeneration via hypocotyl explants

0.5-1.0 cm explants obtained from 6-day old rapeseed seedling hypocotyls have proven optimal for callus production (Cardoza & Stewart, 2003; Rahnama & Sheykhhasan, 2016). The cotyledons were separated to ensure the absence of meristem explants. The callus was formed at the cut sites on the third day of cultivation on MSC medium (Table 1). With increasing of pre-cultivation time on MSC medium up to 1 week, further regeneration occurred by rhizogenesis.

Search for transformation conditions for Ukrainian breeding *Bn1* line of winter rape

For transformation of commercial rapeseed hypocotyl explants agrobacterial suspension is used with an optical density at a wavelength of 600 nm (OD_{600}) from 0.2 (Maheshwari et al., 2011), 0.4 (Hussain et al., 2014), 0.5 (Liu et al., 2015) to 0.8 (Mashayekhi et al., 2008) and 1.0 (Hao et al., 2010). Currently the influence of inoculation time on the regeneration and transformation frequency of winter commercial rape lines is not studied yet. To determine the optimal bacterial cell density the following values OD_{600} were used: 0.2, 0.5 and 1. The inoculation time was 10, 30 and 60 min. The control was considered the regeneration frequency of winter rapeseed explants *Bn1* line without inoculation and selection. During the experiments, it was determined that the optical density of bacteria OD_{600} = 0.5 is optimal for inoculation of rapeseed explants, since the regeneration frequency after transformation is significantly reduced at OD_{600} = 0.2 and OD_{600} = 1.0. In addition, at OD_{600} = 1.0, the agrobacterial contamination and necrosis formation frequency increases after co-cultivation (Figure 2).

The maximum rate of explant regeneration (25.02% ± 4.59%) after transformation was observed with tissue inoculation time 10 min (Figure 3). Increasing of inoculation time contributed to the significant necrosis and contamination of tissues, which made the process of obtaining regenerated plants more complicated (Figure 3).

Often, to increase the *Agrobacterium*-mediated transformation frequency, acetosyringone (50-200 μM), a synthetic phenolic compound that activates *A. tumefaciens* virulence genes, is added into the inoculation medium (Boulter et al., 1990; Cardoza & Stewart, 2003; Mashayekhi et al., 2008; Hao et al., 2010; Maheshwari et al., 2011; Hussain et al., 2014; Liu et al., 2015; Rahnama & Sheykhhasan, 2016; Ravanfar et al., 2017). It was established, that the addition of acetosyringone to the inoculation medium is not appropriate because it causes necrosis of rapeseed tissues and reduces the winter rape *Bn1* line regeneration frequency by 20%.

Step-by-step selection with increasing of phosphinothricin concentration in the regeneration MSR medium up to 8 mg/l allowed to select the maximum number of transgenic plants and to reject false-positive variants. In order to form roots by the plants, the ppt concentration was reduced to 3 mg/l in MSE and MSA media.

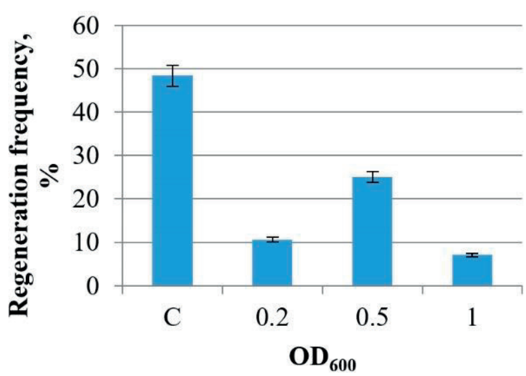

Figure 2. Dependence of the regeneration frequency of winter rapeseed *Bn1* line on the selective MSR medium on the optical density of the bacterial suspension OD at a wavelength 600 nm (with 10 min inoculation); C - Control

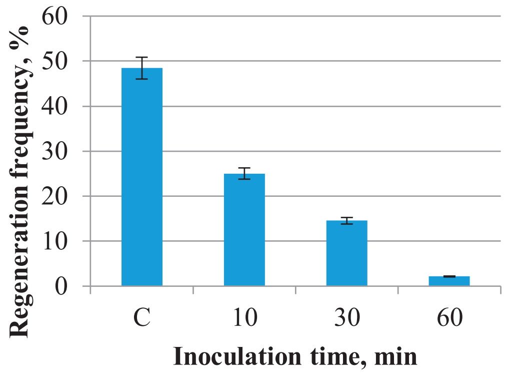

Figure 3. Dependence of the regeneration frequency of winter rapeseed *Bn1* line on the selective MSR medium on the inoculation time (optical density of bacterial suspension OD_{600} = 0.5); C - Control

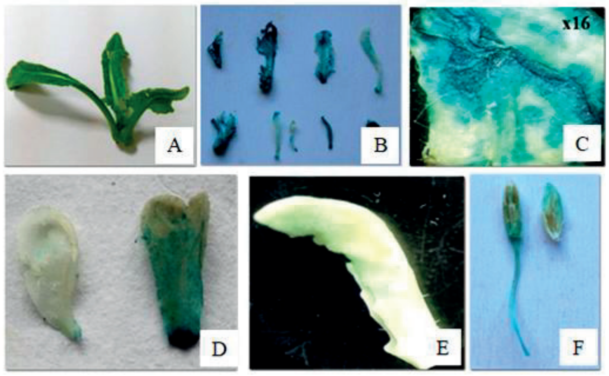

Figure 4. Histochemical analysis of β-glucuronidase expression in plant tissues:

A - winter rape *Bn1* line regenerated plant, obtained after genetic transformation using the pCB203 construct; *B, C, F* - the β-glucuronidase expression in leaves and buds of transformed rapeseed; *D* - negative and positive control (*Nicotiana tabacum* L.); *E* - negative control (*Brassica napus* L.)

Checking the marker gene stable integration

Histochemical analysis of GUS activity revealed the β-glucuronidase expression in regenerated plants and in all fertile winter rapeseed plants of *Bn1* line (Figure 4).

PCR by the *gus* gene confirmed the presence of the transgene in 20 of the 22 rapeseed plants.

Thus, the transformation rate of winter rapeseed *Bn1* line is 22.75% ± 2.28% (Figure 5).

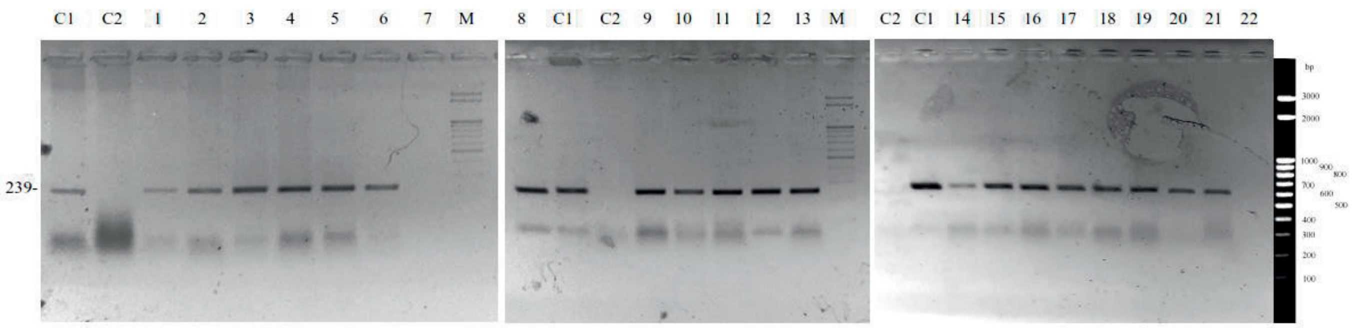

Figure 5. PCR analysis of transgenic rape plants using *gus* specific primers:

Lanes 1-22 - test samples; *C1* - positive control - *Agrobacterium tumefaciens* GV3101 pCB203 colony; *C2* - negative control - intact rapeseed *Bn1* line DNA; *M* - a molecular marker DNA LadderMix

Winter rapeseed *Bn1* line transgenic seeds obtaining

After rooting on MSA medium all regenerated winter rapeseed *Bn1* line plants were planted in peat mixture and vernalized by (Savelieva & Tarakanov 2014; Filek et al., 2007; Waalen et al., 2014). Seeds were obtained from vernalized winter plants (Figure 6).

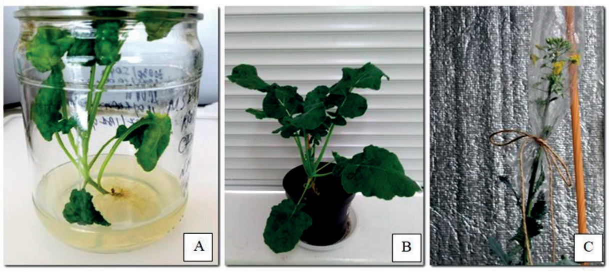

Figure 6. Transgenic seeds of winter rapeseed Bn1 line obtaining after transformation by pCB203 vector:
A - rooting of regenerated plants on the MSA medium;
B - adaptation of plants to the *in vivo* conditions;
C - isolation of peduncles after vernalization for seeds obtaining

CONCLUSIONS

This study presents an optimized protocol for *Agrobacterium*-mediated genetic transformation of Ukrainian breeding winter rape. The aim of transformation was to choose the optimal inoculation time with the agrobacterial suspension, suspension optical density and the composition of the inoculation and regeneration media. We selected following parameters: the optical density of the bacterial suspension for plant inoculation should be $OD_{600} = 0.5$, and the inoculation time - 10 min. The addition of acetosyringone caused a negative effect on the rapeseed regeneration rate in our experiments. Also in our work we used 2-iP as a cytokinin instead of Zeatin, which is 5 times more expensive than the first one. According to the PCR results, the transformation frequency of the *Bn1* line by the *gus* gene is 22.75% ± 2.28%. After vernalization of rapeseed plants seeds, that do not morphologically differ from native, were obtained. Thus, this technique can be used to obtain biotechnological winter rape plants and further research.

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EFFECT OF FERTILIZATION AND STORAGE CONDITIONS ON THE QUALITY OF LETTUCE

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Abstract

This experiment was carried out in 2019, in the cold greenhouse, covered with polycarbonate, in the early culture, in the experimental field of the Faculty of Horticulture Bucharest, and the lettuce was stored in the Postharvest Technology Laboratory at the Research Center for Studies of Food Quality and Agricultural Products of the University of Agronomic Sciences and Veterinary Medicine Bucharest. F1 lettuce hybrids were used: Centore, Analena, Alanis, Shangore and Tizian, fertilized at planting with Best starter 200 kg/ha, K-sol 80 kg/ha, Novatec classic 300 kg/ha and Orgevit 3000 kg/ha. For storage only 2 hybrids, Tizian and Shangore, were used with 2 fertilizers, respectively, Best starter and K-sol. The storage conditions were: in the normal atmosphere at 1°C, 95% humidity and 20% oxygen and in the controlled atmosphere at 1°C, 95% humidity and 2% oxygen for 14 days. There were used 3 packaging variants: unpacked, packaged in food foil and packaged in food foil on cardboard. Plant growth and production were influenced by the fertilizer used, the best results being obtained by fertilizing with Best starter and K-sol, which is why these hybrids were chosen for storage. During the storage period it was observed that the greatest weight loss was in the unpackaged lettuce both in the normal and controlled atmosphere, regardless of the hybrid and the fertilization product used. The content in vitamin C was higher in hybrids fertilized with K-sol, but during the storage period there was a strong decrease especially for the lettuce from stored in controlled atmosphere, for all packaging variants. The acidity of the lettuce did not show major differences.

***Key words**: fertilizer, humidity, oxygen, production, storage, temperature.*

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the most important species amongst the green vegetables. The quality of the edible part depends on the culture system, the applied technology and the biological material used. Lettuce is little pretentious to atmosphere al factors, which makes it possible to cultivate these plants throughout the year, in Romania, in different culture systems to ensure fresh and qualitative lettuce to the consumer market from the national production. An important part in lettuce culture is played by the variety or the hybrid that is cultivated, which is why new hybrids, with superior characteristics regarding productivity and ability to adapt to the atmosphere, are more and more cultivated, especially within protected spaces (Neață et al., 2017). Applying certain products during the vegetation period, for some species, stimulates the growth of the root system and increase the capacity to absorb water and minerals (Helepciuc et al., 2019), while applying some stimulants during the vegetative growth period leads to intensifying some physiological processes, with effects on increasing production and quality (Dobrescu et al., 2017).

Lettuce contains a large variety of phyto-nutrients, fibres and other elements necessary to a proper functioning of the human body (Hoy et al., 2019) and it is included in the food diets along other vegetables and fruits. Lettuce leaves are rich in calcium, iron, magnesium, potassium, folic acid, vitamin K, vitamin C, lutein, beta-carotene, lycopene and other elements essential for the human body. Consuming lettuce and other vegetables with green leaves reduces the incidence of some severe diseases, such as cardiovascular conditions (Pollock, 2016), strokes (Larsson, 2013), cancer (Sant et al., 2007) and others (Padayatty et al., 2003). Fresh products can represent means to transmit pathogens that lead to consumer diseases, which is why packing the products is necessary. Research conducted by Oliveira et al. (2010), on lettuce packed in different types of wrap, stored at 5°C and 25°C, and 10 days after at 5°C, showed that the populations of *E. coli* and *Salmonella* spp. Decreased, while the populations of *Listeria monocytogenes* increased. The 0°C temperature during lettuce storage ensured that the quality was maintained, compared to the temperature of 20°C, but is was also managed to store lettuce in a good condition at 20°C and relative humidity of 85-95% by applying a treatment with 0.2 µl/l 1-MCP (methylcyclopropene) or giberelic acid 0.1 mg/l GA (Tian et al., 2014).

Lettuce is a perishable lettuce, which is why it is recommended to be consumed fresh, but it can also be stored at 4-5°C for several days. Extending the storage period even in optimum conditions leads to an accelerated decrease in vitamin C content (Dewhirst et al., 2017). Reasearch conducted by Spinardi and Ferrante (2012) showed that for the lettuce stored at temperatures of 4 and 10°C the loss in vitamin C was very high, it actually almost disappeared after 5 days since storage, regardless of the temperature, stating that that could be a biochemical marker of the freshness of the lettuce. The lettuce chopped and packed in bags, stored at 2°C, kept its quality 2.5 times more than the one stored at 10°C, as per Bolin et al. (1977).

Manzocco et al. (2017) studied the evolution of some quality parameters of the packed lettuce, at 4°C, 8°C and 12°C, and noted that with the increase in temperature, there was no effect of lettuce firmness, no weight loss, but the colour changed, microbial infections appeared, which led to being impossible to capitalize the product. Increasing the temperature and the level of CO_2 in storage spaces determines the degradation of product quality by altering the content in some nutrients (Tilahun et al., 2017; Kapetanakou et al., 2019). The change in the green colour for some vegetable species, due to the alteration of the chlorophyll and accumulation of carotenoids and/or flavones, was also observed by Kulaj (2015). Applying organic fertilizers determines the increase of the productive yield and lettuce quality compared to the ones obtained by applying chemical fertilizers. Thus, Chen Bo-Ching et al. (2014), showed that fertilizing the lettuce with organic products, 200 kg/ha, led to obtaining lettuce plants with a lower content in nitrates and with a higher growth, compared to fertilizing the plants with the same quantity of chemical products. Hossain and Ryu (2018), showed that using organic fertilizers for lettuce determined an increase in production and its content in different nutrients, an increase the soil content in nitrogen and organic matter and a decrease in heavy metals content of the soil (cadmium and lead).

MATERIALS AND METHODS

Research was conducted within 2 experiments, the first one for producing lettuce and the second one for storing it.

Experiment 1

The lettuce culture was established in the experimental field of the Faculty of Horticulture of Bucharest, during 2018-2019, for the spring culture, within an unheated greenhouse, covered with double-layered polycarbonate and with protection against ultraviolet rays. The experiment had two factors, 5 hybrids and 4 products for soil fertilization were used, during the soil preparation for planting, as detailed below:

Factor A Hybrid	Factor B Fertilizer
a1- Tizian F1 a2 - Shangore F1 a3 - Alanis F1 a4 - Analena F1 a5 - Centore F1	b1 - Best Starter, 200 kg/ha b2 - K Sol, 80 kg/ha b3 - Novatec clasic, 300 kg/ha b4 - Orgevit, 3000 kg/ha

The purpose of the experiment was to determine the influence of the hybrid on lettuce production, the influence of the fertilizer on the growth of edible parts and the combined influence of the two factors on the growth and productive yield of the lettuce cultivated in unheated greenhouse. The culture was established in the first decade of March, with 40-day-old seedlings. Planting was done at 40 cm between rows and 30 cm between plants on a row, resulting around 8 plants/m^2. The fertilization was made through dispersal and soil incorporation, on different variants. Specific maintenance works were applied to the culture. Harvesting of the lettuce was done 38 days after planting, moment in which physical-chemical measurements were made.

Experimental design

The experiment was organized into subdivided parcels, with 3 replications. Each replication had 5 plants for which measurements were made at harvest. Measurements regarding plant weight were made through weighing with the electronic scale WTB 2000, while for determining the root system volume the graded cylinder was used.

The interpretation of the results was made through variance analysis, the values being compared to the average of the experiment.

Experiment 2

It was conducted at the Research Center for Studies of Food Quality and Agricultural Products - Hortinvest from the USAMV Bucharest. Two hybrids were stored in controlled atmosphere, Shangore F1 and Tizian F1, respectively, obtained from a culture fertilized Best starter (BS) and K-sol (KS). The purpose was to establish the effect of storage conditions on lettuce quality. The lettuce was stored in normal atmosphere conditions (CR), at a temperature of 1°C, humidity of 95%, 20% oxygen level, and controlled atmosphere (CA), at a temperature of 1°C, humidity of 95%, 2% oxygen, for 14 days. The lettuce was individually packed in food wrap, food wrap + tray (cardboard plate for support) and unpacked.

Measurements made

Three measurements were made: the first at the beginning, immediately after harvest, when the lettuce was introduced to storage, and others after 7 and 14 days. The following were measured: vitamin C (Chanforan et al., 2012; Turmanidze et al., 2017), using the tool Agilent Technologies 1200 equipped with detector UV-DAD through column ZORBAX Eclipse XDB-C18 (4.6 x 50 mm, 1.8 pm id), and the data was recorded and processed using the software Agilent Chem Station B.04.03 (Agilent, USA); acidity was measured with the automatic titrator TitroLine easy. It was titrated with NaOH 0.1 N, until a pH value of 8.1 (Saad et al., 2014; AOAC Official Method, 942.15) and was calculated using the formulae:

Titrable acidity (%) = (V x N x C x 100)/m

V=volume of NaOH consumed; N=normality of NaOH; C=malic acid equivalent; m=sample mass; losses recorded during storage, through weighing; content in soluble dry matter using the refractive method with digital refractometer (Kruss DR301-95) and total dry matter by drying in the drying oven at 105°C.

RESULTS AND DISCUSSIONS

The influence of the hybrid and fertilizer on the growth of lettuce plants

The results obtained after interpreting the data recorded during the experiment showed that both the hybrids and the used fertilizers had a very good influence on lettuce growth and productivity (Table 1). The weight of the edible part was influenced by all used fertilizers, but the best results were recorded for the fertilizer K-sol with 4 of the hybrids: Tizian F1, 375.9 g, Centore F1, 384.4 g, Shangore F1, 405.4 g and Analena F1, 416.7 g. The weight of the non-edible part had values between 7.3 g for the hybrid Analena F1 fertilized with Best starter and 29 g for the hybrid Tizian F1 fertilized with K-sol, but without noticing a certain influence of the hybrid or the fertilizer used. The root system of lettuce plants had similar weight values for all studied hybrids, between 16.8 g and 19.2 g, except for the hybrid Centore F1 for which the roots had values between 11.7 g and 14.6 g. However, root volume was more uniform and no great variations were recorded amongst the studied variants. The lettuce production is similar to the production obtained in Romania (Horgos et al., 2001), in unheated spaces, and had values between 2.02 kg/m^2 for Alanis F1 fertilized with Novatec classic and 3.33 kg/m^2 for Analena F1 fertilized with K-sol. For the majority of the studied combinations, the largest productions were obtained as a result of the fertilization with Best starter and K-sol.

Table 1. Biometric characteristics of lettuce plants at harvest

Combination	Weight of edible part (g)	Weight of non-edible part (g)	Root		Production (kg/m^2)
			Root weight (g)	Root volume (cm^3)	
a1b1	368.8	28.5	16.8	22	2.95 N
a1b2	375.9	29.0	19.1	24	3.01**
a1b3	306.2	27.0	18.0	18	2.45^{000}
a1b4	339.5	17.5	19.2	20	2.72 N
a2b1	397.7	24.1	17.4	18	3.18***
a2b2	405.4	22.9	19.0	22	3.24 ***
a2b3	342.8	24.5	18.2	24	2.74 N
a2b4	344.1	21.7	19.2	16	2.75 N
a3b1	366.1	8.3	16.8	16	2.93 N
a3b2	256.7	8.2	17.4	22	2.05^{000}
a3b3	252.3	16.0	18.0	20	2.02 ^{000}
a3b4	362.3	12.3	19.2	18	2.90 N
a4b1	371.3	7.3	18.8	16	2.97*
a4b2	416.7	11.3	18.0	26	3.33***
a4b3	365.0	14.3	18.0	22	2.92 N
a4b4	347.3	9.7	18.4	20	2.78 N
a5b1	361.2	11.4	12.6	21	2.89 N
a5b2	384.4	17.8	14.6	22	3.08 ***
a5b3	364.6	12.3	13.8	19	2.92 N
a5b4	353.5	11.2	11.7	20	2.83 N
LSD 5%					0.12 kg/m^2
LSD 1%					0.16 kg/m^2
LSD 0.1%					0.22 kg/m^2

The influence of the packing variant and storage duration on lettuce quality in normal atmosphere

Lettuce is a highly perishable vegetable, which is why it is recommended for consumption immediately after harvest or for storage in certain conditions to maintain qualitative characteristics, except for vitamin C (Dewhirst et al., 2017). Regarding the content in vitamin C, it was observed that for the fertilization with K-sol, the obtained plants had a higher content in vitamin C (5.38-7.69 mg/100 g fresh lettuce) compared to the plants fertilized with Best starter (4.43-4.89 mg/100 g fresh lettuce). After 7 days, the content in vitamin C decreased more for the unpacked lettuce, fertilized with K-sol (41.08% for Tizian F1 and 58.00% for Shangore F1) compared to the one fertilized with Best starter, where the loss was of 13.70% for Tizian F1 and 20.31% for Shangore F1. For the lettuce packed in food wrap or food wrap+cardboard, the loss was generally higher than for the unpacked lettuce for both hybrids except for Shangore F1 fertilized with K-sol, packed in food wrap, for which the loss was of 50.59%. After 14 days, the greatest loss was recorded also for the packed variants. Packing the lettuce determined the highest loss in vitamin C, the values being between 74.49% for Shangore F1+BS and 92.85% for Shangore F1+KS (Table 2). These results are in according with other results from speciality literature (Spinardi and Ferrante 2012; Bolin et al., 1977).

Within the controlled atmosphere, the loss in vitamin C were larger after 7 days compared to the normal atmosphere, for all packing variants, for all experimental variants, with values between 71.98% for Tizian F1+BS, unpacked, and 90.9% for Shangore F1+KS, packed in food wrap + cardboard (Table 3).

Table 2. Vitamin C content of the lettuce stored in normal atmosphere

Time of analysis (days)	Packing variant	Hybrid + fertilizer							
		Shangore F1+BS		Shangore F1+KS		Tizian F1+BS		Tizian F1+KS	
		mg/100 g	%	mg/100 g	%	mg/100 g	%	mg/100 g	%
0		4.43	0	7.69	0	4.89	0	5.38	0
7	N	3.53	20.31	3.23	58.00	4.22	13.70	3.17	41.08
	F	2.38	46.27	3.80	50.59	1.79	63.39	2.12	60.59
	F+C	2.14	51.69	1.56	79.71	1.75	64.21	1.37	74.54
14	N	2.03	54.17	2.40	68.80	4.78	20.25	3.14	41.64
	F	1.13	74.49	0.55	92.85	0.82	83.23	0.59	89.03
	F+C	0.66	85.10	0.55	92.84	0.57	88.34	0.79	85.31

After 14 days, the vitamin C loss was slightly higher than after 7 days, with values between 84.76% for Tizian F1+KS, unpacked, and 94.15% for Shangore F1+KS, packed in food wrap + cardboard, very high values compared to the when the lettuce was introduced to storage. The packing variant had a very low influence on the loss of vitamin C during storage.

Table 3. Vitamin C content of the lettuce stored in controlled atmosphere

Storage condi-tions	Hybrid + fertilizer									
	Time of analysis (days)	Pac-king variant	Shangore F1+BS		Shangore F1+KS		Tizian F1+BS		Tizian F1+KS	
			mg/100 g	%	mg/100 g	%	mg/100 g	%	mg/100 g	%
	0		4.43	0	7.69	0	4.89	0	5.38	0
CA	7	N	0.84	81.04	1.51	80.36	1.37	71.98	0.76	85.87
		F	0.73	83.52	0.75	90.25	0.77	84.25	1.13	79.00
		F+C	0.46	89.62	0.7	90.90	0.76	84.46	1.05	80.48
	14	N	0.64	85.55	0.57	92.59	0.55	88.75	0.82	84.76
		F	0.56	87.36	0.63	91.81	0.52	89.37	0.65	87.92
		F+C	0.50	88.71	0.45	94.15	0.52	89.37	0.44	91.82

Lettuce acidity had values of 0.07-0.08% malic acid when stored, the values slightly increasing as the storage duration increased, regardless of the packing variant and the storage conditions (Table 4). The highest value of acidity was recorded after 14 days of storage, for Shangore F1 with both fertilizers, for unpacked lettuce, 0.13-0.14%.

The lettuce content in soluble dry matter (Table 5) immediately after harvest had values between 1.97% and 3.00% for the hybrid Shangore F1 and 2.27-2.30 for the hybrid Tizian F1, with both fertilizers.

In normal atmosphere, for the unpacked lettuce, after 7 days of storage, the content in soluble dry matter increased compared to the initial moment, except for the hybrid Shangore F1+KS for which it decreased. After 14 days, the content decreased compared to the initial moment, except for the hybrid Shangore F1+BS for which it slightly increased (2.10%). For the packed lettuce, the content in soluble dry matter increased after 7 days for all studied variants compared to the initial moment, while after 14 days of storage it decreased below the initial value, except for the hybrid Shangore F1+BS for which it increased (2.50%).

In controlled atmosphere, for the unpacked lettuce, after 7 days of storage, the values increased for Shangore F1+BS and Tizian F1+KS and decreased for the other two variants. For the packed lettuce, no clear influence of the hybrid, storage duration or packing variant could be observed, the values for this parameter being somehow irregular.

The lettuce content in total dry matter (Table 6) recorded values between 4.17% and 5.11% at harvest and introduction to storage. After 7 days of storage, it was observed that the values increased to 6.53% for Shangore F1+BS, packed in food wrap + cardboard, and to 6.19% for Tizian F1+BS, unpacked. It was observed that for the unpacked lettuce, for the majority of the studied variants, the content in total dry matter was higher compared to the packed lettuce, for which the water loss through evapo-transpiration were lower. The lowest content in total dry matter was recorded for Shangore F1+BS, packed in food wrap, 3.38%, after 14 days of storage.

Table 4. Titrable acidity (% malic acid)

Storage conditions	Time of analysis (days)	Packing variant	Hybrid + fertilizer Shangore F1+BS	Shangore F1+KS	Tizian F1+BS	Tizian F1+KS
	0		0.07	0.08	0.07	0.08
NA	7	N	0.11	0.1	0.11	0.1
		F	0.09	0.1	0.06	0.08
		F+C	0.09	0.09	0.08	0.08
	14	N	0.13	0.12	0.08	0.1
		F	0.1	0.08	0.11	0.09
		F+C	0.11	0.12	0.07	0.11
CA	7	N	0.1	0.08	0.07	0.07
		F	0.08	0.1	0.09	0.08
		F+C	0.09	0.08	0.08	0.09
	14	N	0.14	0.13	0.11	0.11
		F	0.09	0.1	0.08	0.1
		F+C	0.1	0.11	0.07	0.09

Table 5. Soluble dry matter content of the lettuce (%)

Storage conditions	Time of analysis (days)	Packing variant	Hybrid + fertilizer Shangore F1+BS	Shangore F1+KS	Tizian F1+BS	Tizian F1+KS
	0		1.97	3.00	2.30	2.27
NA	7	N	2.43	2.23	2.63	2.90
		F	3.07	3.07	2.57	2.87
		F+C	3.10	3.27	3.20	2.83
	14	N	2.10	1.83	1.23	1.83
		F	1.63	1.67	2.00	1.67
		F+C	2.50	1.97	2.03	1.83
CA	7	N	2.47	2.70	1.40	2.37
		F	2.80	2.43	2.80	2.53
		F+C	2.27	2.45	2.53	2.67
	14	N	2.23	2.57	2.63	2.30
		F	2.13	2.50	1.83	1.43
		F+C	2.70	2.23	1.33	1.73

The weight loss of the salad, especially the packaged, kept at 4-12°C are very small, says Manzocco et al. (2017), but changes in color or microbial infections occur.

Analyzing the weight loss of the lettuce during storage (Table 7), it was noted that the highest loss was recorded for the unpacked lettuce, both for the storage in normal atmosphere and controlled atmosphere. The values of the weight loss for the unpacked lettuce stored within normal atmosphere was between 10.00% for Shangore F1+KS and 12.53% for Tizian F1+K-sol, and between 17.33% and 21.41% for the same hybrids, after 14 days of storage. In the case of unpacked storage within controlled atmosphere, after 7 days, the loss was between 7.96% for Shangore F1+Best starter and 9.99% for Tizian F1+K-sol, while after 14 days the loss had values between 14.63% and 21.94%, for the same hybrids. Smaller loss values (sub 0.7%) were recorded for the lettuce packed both in food wrap and food wrap + cardboard, for both storage conditions. Thus, after 7 days, for all studied variants, the weight loss was between 0.02% and 0.12%, while after 14 days it was between 0.04% and 0.64%. To reduce weight loss during storage, lettuce must be packed in order to maintain the water within the tissues.

Table 6. Total dry matter content (%)

Storage conditions	Time of analysis (days)	Packing variant	Hybrid + fertilizer Shangore F1+BS	Shangore F1+KS	Tizian F1+BS	Tizian F1+KS
	0		5.11	5.06	4.17	5.11
NA	7	N	5.29	5.87	6.19	4.98
		F	4.02	4.47	4.40	4.72
		F+C	6.53	4.44	5.63	4.70
	14	N	4.41	4.51	4.15	3.60
		F	3.49	3.99	4.16	4.23
		F+C	3.96	4.04	4.15	4.05
CA	7	N	4.56	5.22	5.73	5.37
		F	4.71	4.28	4.73	4.81
		F+C	3.49	3.71	4.45	4.25
	14	N	4.00	5.16	5.30	5.07
		F	3.38	3.98	3.78	3.61
		F+C	3.61	3.67	3.83	3.84

Table 7. Lettuce weight loss during storage (g)

Storage conditions	Packing variant	Time of analysis (days)	Hybrid + fertilizer Shangore F1+BS (g)	Losses (%)	Shangore F1+KS (g)	Losses (%)	Tizian F1+BS (g)	Losses (%)	Tizian F1+KS (g)	Losses (%)
NA	N	0	**327.88**	**0.00**	**351.15**	**0.00**	**317.64**	**0.00**	**354.39**	**0.00**
		7	294.95	10.04	316.05	10.00	283.94	10.61	310.00	12.53
		14	263.04	19.78	290.29	17.33	258.62	18.58	278.51	21.41
	F	0	**269.95**	**0.00**	**460.92**	**0.00**	**333.92**	**0.00**	**349.69**	**0.00**
		7	269.62	0.12	460.78	0.03	333.63	0.09	349.59	0.03
		14	268.92	0.38	459.92	0.19	331.50	0.64	348.99	0.20
	F+C	0	**331.04**	**0.00**	**434.53**	**0.00**	**292.35**	**0.00**	**348.14**	**0.00**
		7	330.90	0.04	434.44	0.02	292.28	0.02	347.86	0.08
		14	330.60	0.13	434.22	0.07	292.11	0.08	347.72	0.12
AC	N	0	**354.80**	**0.00**	**426.14**	**0.00**	**325.74**	**0.00**	**328.17**	**0.00**
		7	326.55	7.96	385.11	9.63	296.81	8.88	295.40	9.99
		14	302.90	14.63	354.12	16.90	261.45	19.74	256.17	21.94
	F	0	**341.43**	**0.00**	**433.68**	**0.00**	**255.88**	**0.00**	**429.50**	**0.00**
		7	341.33	0.03	433.57	0.03	255.79	0.04	428.21	0.30
		14	341.20	0.07	433.46	0.05	255.58	0.12	427.86	0.38
	F+C	0	**310.61**	**0.00**	**399.42**	**0.00**	**267.15**	**0.00**	**385.40**	**0.00**
		7	310.42	0.06	399.21	0.05	266.84	0.12	385.31	0.02
		14	310.33	0.09	399.03	0.10	266.76	0.15	385.23	0.04

CONCLUSIONS

As a result of the research conducted, it could be seen that in order to obtain vigorous plants and with a higher productive capacity both organic and chemical fertilizers can be used. The best results from this point of view were obtained by using the fertilizers Best starter and K-sol in the quantity recommended by the producer, for 3 of the studied hybrids, Shangore, Tizian and Analena, respectively. Packing the lettuce strongly reduced the weight loss during storage both within normal and controlled atmosphere, for 7 and 14 days, the loss being under 0.7%, which allows the storage of this species for 14 days with the studied atmosphere al conditions. For the unpacked lettuce, for both storage conditions, the weight loss was significantly larger, respectively 7.96 % after 7 days and 21.94 % after 14 days. Lettuce acidity increased slightly with the increase of the storage duration compared to the initial moment.

The level of vitamin C, within normal atmosphere, decreased significantly after 7 days, 58.00% for the unpacked lettuce, and 79.71% for the packed lettuce, respectively 20.25% for Tizian F1+BS and 92.8% for Shangore F1+KS, after 14 days. Within controlled atmosphere, the loss was more uniform and without large differences between the values after 7 days and after 14 days, but very large compared to the initial moment. This experiment showed that lettuce losses the greatest part of vitamin C during storage, regardless of the storage conditions and packing variants. The lettuce content in soluble dry matter and total dry matter recorded different values, without noticing a more significant influence of one of the factors.

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A NEW EYE DROP FORMULATION USED IN THE MANAGEMENT OF CORNEAL ULCERS IN DOGS AND CATS

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Abstract

Corneal ulcers in dogs and cats are very common. Regardless of etiology (melting or indolent corneal ulcers) and of the affected layers of the cornea (superficial or deep corneal ulcers), local treatment is performed using eye drops with antibiotic and corneal healing drugs (hyaluronic acid and acetylcysteine). Each eye should be instilled 5-8 times per day for 14-21 days. In Romania, the eye drops available on the market have only one active substance and the owner must apply the topical medication from several vials. To remove this drawback, a new healing eye drop formulation containing sodium hyaluronate, acetylcysteine and insulin (as a growth tissue factor) has been developed, reducing the number of daily administrations. This new formulation is well tolerated and the corneal healing is achieved by only 3-4 administrations per day.

Key words*: cat, corneal ulcer, dog, eye drops.*

INTRODUCTION

Corneal ulcers are very common encountered in veterinary practice. Depending on the number of affected corneal layers, they can be superficial or deep. Superficial corneal ulcers (Figure 1) are characterized by loss of corneal epithelium and exposure of corneal stroma without stromal loss (Gelatt et al., 2013; Eaton et al., 2017). Deep corneal ulcers involve stromal defect.

Clinically, the patients present with discomfort manifested as blepharospasm, conjunctival hyperemia, ephiphora, photophobia, corneal edema and possibly reflex uveitis (miosis and aqueous flare). A corneal ulcer is diagnosed based on these clinical signs and on the fluorescein uptake by the corneal stroma (Gelatt et al., 2013).

Corneal ulcers can have a wide variety of etiologies: trauma, entropion, ectopic cilia, trichiasis, distichiasis, quantative or qualitative tear film abnormalities, foreign bodies, spontaneous chronic corneal epithelial defects (SCCED), microbial infection, facial nerve paralisys. Corneal sensitivity and aqueous tear production are lower in brachycephalic dogs. Dogs with nasal folds are nearly five times more likely to be affected by corneal ulcers than those without (Packer et al., 2015; Bolzanni et al., 2020).

Figure 1. OD Two-years-old DSH with superficial corneal ulcer

Indolent ulcer or spontaneous chronic corneal epithelial defect (SCCED) is a chronic superficial corneal ulceration with no identifiable cause or complicating factor. It is diagnosed most often in middle-aged to older dogs, and the Boxer breed is overrepresented (Bentley, 2005; Eaton et al., 2017). It is bordered or partially covered with non-adherent epithelium, and fails to heal within a normal time period (Ledbetter et al., 2006) (Figures 2 and 3).

Figure 2. OS Seven-years-old French Bulldog with indolent corneal ulcer, bordered by a lip of non-adherent epithelium

Figure 3. OS Previous case after cotton-tipped swab debridement

A melting ulcer is characterized by rapid and progressive stromal loss and it is potentially globe threatening (Gelatt et al., 2013).
Corneal ulcers' standard treatment consists of topical antibiotics, corneal healing drugs and debridement in SCCED. Cases with deep corneal ulcer (Figure 4) may require surgery that may include application of a collagen bandage lens or amniotic membrane, third eyelid flap, keratectomy in SCCED, conjunctival grafts (Gelatt et al., 2013; Ionascu & Ion, 2013; Ion et al., 2016).
In Romania, the eye drops available on the market have only one active substance and the owner must apply the topical medication from several vials. Owner compliance is an important factor in the success rate of the treatment. Some owners find it difficult to administer eye drops 5-8 times daily, from 2-3 different bottles, especially if the patient is aggressive.

Figure 4. OD Twelve-years-old Crossbred with deep corneal ulcer

To remove this drawback, a new healing eye drop formulation (ii-2018) containing sodium hyaluronate, acetylcysteine and insulin (as a growth tissue factor) has been developed, reducing the number of daily administrations.
Insulin has been shown to improve corneal epithelial healing *in vitro* and in diabetic animal models (Nagano et al., 2003; Wang et al., 2017). However, clinical experience with topical insulin in patients with corneal wounds is minimal (Wang et al., 2017; Ghiasi et al., 2018).
The aim of this study is to test the clinical efficacy of the ii-2018, a new healing eye drop formulation.

MATERIALS AND METHODS

To determine the efficacy of this new eye-drop formulation (ii-2018), a total of 102 dogs and 35 cats diagnosed with superficial or deep ulcers were enrolled in this study.
Prior to ophthalmic examination, affected eyes were topically anesthetized with oxybuprocaine hydrochloride 0.4% (Benoxi®, Unimed Pharma).
Dogs underwent ophthalmic examinations and 42 were diagnosed with indolent ulcer (SCCED) (Figures 5 and 6), 28 with deep corneal ulcers and 32 dogs with melting ulcer.
All dogs with indolent ulcer underwent a sterile cotton-tipped swab debridement. The cotton-tipped swab was passed over the ulcer in multiple circular passes, removing the non-adherent epithelium.

Figure 5. OS Seven-years-old French Bulldog with indolent corneal ulcer, clinical aspect after debridement

Figure 6. OS Eight-years-old English Bulldog with indolent corneal ulcer, clinical aspect after debridement

A total of 35 cats were included in this study. They underwent complete ophthalmic examination and 8 cats were diagnosed with superficial corneal ulcer (Figure 7), 15 with deep corneal ulcer (Figure 8) and 12 cats with melting ulcer (Figure 9).

Figure 7. OD Nine-months-old BSH with large superficial corneal ulcer

Figure 8. OS Three-years-old DSH with deep corneal ulcer

Figure 9. OD Eight-years-old Persian with melting corneal ulcer

All patients received ii-2018 and a topical antibiotic (ofloxacin, Floxal, Bausch & Lomb Rochester, NY, SUA), 3 to 4 daily administrations until recovery (7-28 days). Deep and melting ulcers were treated systemically with doxycycline 10 mg/kg SID (Ronaxan 20 mg, Merial, France).

ii-2018 is presented in a 10 ml sterile bottle. The drops were prepared by a pharmacy by mixing insulin (Humulin R 100 UI/ml, Lilly S.A., Madrid, Spania) with 5% acetylcysteine and 0.2% sodium hyaluronate. The drops were refrigerated and used up to 1 month after preparation.

The corneal ulcers were considered healed when the cornea revealed a negative fluorescein test and there was no evidence of blepharospasm or ocular discharge.

Clinical follow-up included the evaluation of potential side effects as well, such as: local inflammation, pruritus, pain, excessive lacrimation.

Data were analyzed for breed, age, additional ocular disorders, duration of clinical signs

before referral, number of days before healing was achieved, number of debridements, additional therapeutic interventions, complications.
Patients were re-checked weekly (+/- 3 days) from the beginning of the treatment for a total of 4 weeks.

RESULTS AND DISCUSSIONS

The corneal ulcers had been treated by referring veterinarians for a median of 15 days (range: 1-40 days) prior to enrolment in this study. Medical treatment consisted of topical +/- systemical antibiotics, topical corticosteroids, artificial tear solutions with hyaluronic acid or dexpantenol.
Dog breeds included crossbreds (n = 28), Shih-Tzu (n = 21), French Bulldog (n = 20), Boxer (n = 10), English Bulldog (n = 9), Pug (n = 7), Caniche (n = 5), West Highland Terrier (n = 2).
The median age was 7 years, with a range between 9 months and 13 years.
From the total number of dog patients (n = 102), 42 dogs (41.17%) were diagnosed with indolent ulcers, 28 dogs (27.45%) with deep corneal ulcers and 32 dogs (31.37%) with melting ulcer.
After 1 week of treatment, 16.66% (7/42) of cases with indolent corneal ulcers healed (Figures 10, 11 and 12).

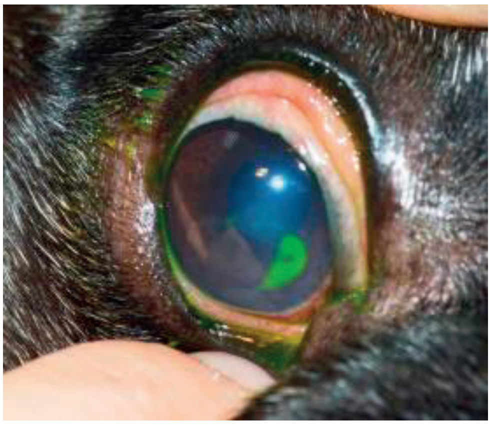

Figure 10. OD Three-years-old French Bulldog with indolent corneal ulcer, clinical aspect at the initial time of presentation

After 2 weeks of treatment, 64.28% (27/42) of cases with indolent corneal ulcer (Figure 13), 28.57% (8/28) of cases with deep ulcer and 31.25% (10/32) of cases with melting ulcer had healed.

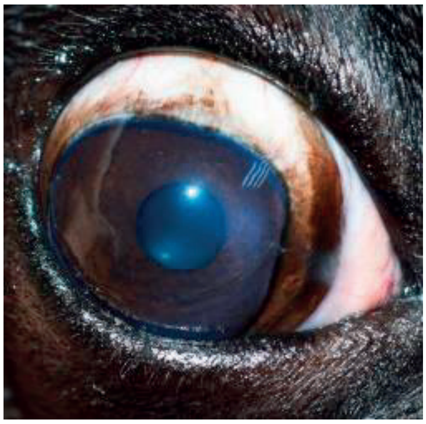

Figure 11. OD Previous case at 7 days re-check examination. The ulcer is healed, mild corneal opacity

Figure 12. OS Seven-years-old French Bulldog with indolent ulcer healed after 7 days of treatment (same case from Figure 3)

Figure 13. OS Seven-years-old French Bulldog with indolent ulcer healed after 14 days of treatment (same case from Figure 5)

At the third recheck examination, 88.09% (37/42) of cases with indolent corneal ulcer, 67.85% (19/28) of cases with deep ulcer and 78.12% (25/32) of cases with melting ulcer had healed.
After 4 weeks of treatment, 92.85% (39/42) of cases with indolent corneal ulcer (Figures 16 and 17), 100% (28/28) of cases with deep ulcer

(Figures 14, 15 and 18) and 93.75% (30/32) of cases with melting ulcer had healed.

Figure 14. OD Seven-years-old Shih Tzu with deep corneal ulcer at the beginning of the treatment

Figure 15. Previous case, seven-years-old Shih Tzu healed at 28 days of treatment

Cases with indolent corneal ulcer had corneal vascularization in 40% of cases at the time of the second examination (Figure 16).

Figure 16. OS Eight-years-old English Bulldog with indolent corneal ulcer, clinical aspect 14 days after the beginning of the treatment (fluorescein test is positive and cornea has neovascularization)

A second debridement was performed in 35.71% (15/42) of cases, based on presence of redundant epithelial tissue at the ulcer's margins. Ulcer healed at 28 days of treatment with superficial corneal scar (Figure 17).

Figure 17. Previous case eight-years-old English Bulldog with indolent corneal ulcer healed at 28 days of treatment with superficial scarring (same case from Figure 6)

Figure 18. OD Twelve-years-old Crossbred with deep corneal ulcer healed at 28 days of treatment (same case from Figure 4). Corneal opacity and mild neovascularization

Systemic antibiotic, doxycycline 10 mg/kg SID (Ronaxan, Merial, France) were prescribed in all brachycephalic cases and in cases where a large corneal area had been debrided.

The 3 cases with indolent ulcer that failed to heal after 4 weeks of therapy were treated with Softshield® bandage collagen lens and a third eyelid flap. After 21 days, at sutures' removal, 100% (3/3) of lesions were healed. The reason for ii-2018 failure in this cases might be the age of the patients (10, 12 and 13 years old), the associated treatment with systemical corticosteroids (for lymphoma and optic neuritis).

Two melting ulcers had a bad outcome after 3, respectively 7 days of treatment and underwent surgical treatment with Softshield® and third eyelid flap as well. After 21 days, at sutures' removal, 100% (2/2) of lesions were healed. Both cases were represented by brachycephalics. In one case, the owner could not administer the eye drops.

Cat breeds represented in this study included Domestic Shorthair (n = 20), British Shorthair (n = 8), Persian (n = 5) and Sphinx (n = 2).

The median age was 6 years, with a range between 7 months and 11 years.

From the total number of cat patients (n = 35), 8 cats (22.86%) were diagnosed with superficial ulcers, 15 cats (42.86%) with deep corneal ulcers and 12 cats (34.28%) with melting ulcer.

All superficial corneal ulcers healed in the first week (3/8) and after 14 days of treatment (8/8) (Figure 19).

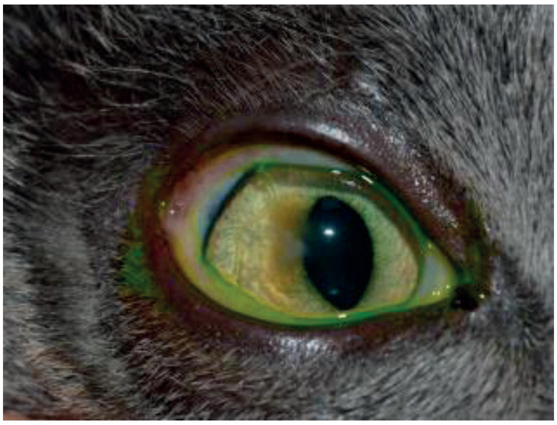

Figure 19. Nine-months-old British Shorthair with large superficial corneal ulcer, clinical aspect at 14 days re-check examination. The fluorescein test is negative (same case from Figure 7)

Four out of fifteen (26.66%) cats with deep corneal ulcer were healed after 14 days of treatment, 11/15 (73.33%) after 21 days, and all cases were healed after 28 days (Figures 20 and 21).

One cat with melting ulcer healed after 14 days. At the third recheck examination, 4/12 (33.33%) with melting ulcer had healed. A total of seven additional cases were resolved at 28 days, bringing the total number of resolved cases to 11/12 (91.66%).

Figure 20. OS Three-years-old Domestic Shorthair with deep corneal ulcer (case from Figure 8) after 14 days of treatment. The fluorescein test is negative, mild corneal scarring, the ulcer is healed

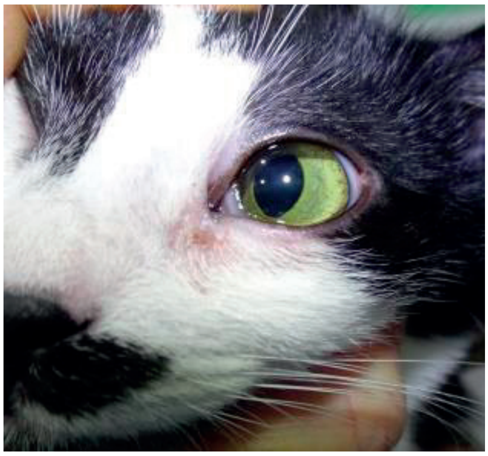

Figure 21. Previous case, at 28 days post initial examination. The cornea is transparent

The complications encountered in this case series were corneal scarring, pigmentation, vascularization, corneal sequestrum, opacification and specific vascularization in cases with concurrent keratoconjunctivitis *sicca*.

In dogs, 18/28 deep ulcers healed with corneal scarring and 25/32 melting ulcers developed post-healing corneal opacity, persistent vascularization (Figure 18) and pigmentation.

In cats, 6/15 with deep ulcer developed corneal scarring, and 9/12 of melting ulcers healed with corneal opacity. Two cases of melting ulcer developed corneal sequestrum 3 weeks, respectively 2 months after healing (Figure 22).

Figure 22. OD Eight-years-old Persian with corneal sequestrum (same case from Figure 9)

This eye drop formulation was developed based on the existing studies in the literature.

Acetylcysteine has mucolytic and anticollagenolytic documented properties and had been used to treat keratoconjunctivitis *sicca*, alkali-burned corneas in rabbits and corneal mucous plaques. 3% acetylcysteine is effective in decreasing the healing time of corneal wounds in dogs (Aldavood et al., 2003).

The effect of topical insulin on corneal lesions has been documented in rodent models. In diabetic rats, topical insulin improves corneal sensation and promotes wound healing after corneal abrasions (Zagon et al., 2007; Wang et al., 2017).

Insulin-like growth factor-1 (IGF-1) has been shown to be an important modulator of corneal wound healing.

In some studies, IGF-1 was shown to act synergistically with substance-P to promote corneal epithelium wound healing (Nagano et al., 2003; Nishida & Yanai, 2009; Wang et al., 2017).

Shanley et al. (2004) suggest a mechanism by which insulin may influence corneal wound healing in vitro (Shanley et al., 2004) and exposure of corneal epithelium to insulin facilitated closure of *in vitro* small wounds through enhanced cell migration instead of proliferation.

Hyaluronic acid is commonly used as part of the treatment in superficial ulcers. Gronkiewicz et al. (2017) found that the viscoelastic properties of hyaluronic acid containing-solutions is also a factor which may be contributing to corneal wound healing.

Hyaluronic acid promotes tear film stability, (Nakamura et al., 2004; Hirai et al., 2005).

Healthy precorneal tear film contains growth factors that play an important role in corneal epithelial wound healing (Klenker et al., 2007; Gronkiewicz et al., 2017). A more recent study demonstrates that a cross-linked, modified HA hydrogel provides further benefit by accelerating time to corneal wound closure compared to a non-cross-linked HA solution (Williams et al., 2017).

The disadvantages of surgical treatment of deep and melting ulcers, include the need for general anesthesia, increased postoperative opacity secondary to corneal scarring and increased costs for the owner. Treatment with ii-2018 is inexpensive, and produces rapid and complete healing in the majority of treated cases.

We must emphasize here the need for careful selection of the patients. If the corneal defect is very deep, down to the Descemet membrane , if the melting ulcer is very aggressive, or if the indolent ulcer does not heal in an acceptable time period, surgical treatment is advised.

Careful monitoring for deterioration of corneal ulcers is warranted when prescribing ii-2018.

Except for mild ocular discomfort manifested with blepharospasm and pruritus (29/137) and mild inflammation of the conjunctiva (1/137), the patients did not show other local or systemic side effects.

The pruritus, the attempt to rub the eye, might be due to the acetylcysteine. The discomfort manifested for less than 10 minutes after administration of ii-2018. The owner compliance is very good, as it is no longer necessary to administer treatment from multiple bottles.

This case series is limited by the heterogeneity of the cases and lack of a comparative control group.

This study did not seek to compare ii-2018 with other treatment option, but to simply determine if it’s safe and effective. Further studies are necessary to compare this new eye drop formulation with other therapies.

CONCLUSIONS

The results of this study suggest that the use of this new eye-drop formulation (ii-2018) may be a safe and effective treatment for corneal ulcers in dogs and cats. This new formulation is well tolerated and the corneal healing is achieved by only 3-4 administrations per day.

Local treatment using a single bottle instead of three is easy for the owner.

Further studies are necessary to determine the clinical efficacy in extented trials.

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MANAGEMENT INDUCED CHANGES IN POND WATER QUALITY AND GROWTH PERFORMANCE OF GOLDFISH, *Carassius auratus* (L.), IN TWO 11-WEEK GROWTH EXPERIMENTS CONDUCTED DURING THE SUMMER AND WINTER SEASONS

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Abstract

To assess the seasonal influence on the growth performance of goldfish, Carassius auratus (L.) in earthen ponds maintained under different production management regimes, two 11-week growth experiments were conducted during two different seasons (summer and winter) under tropical conditions in India. Weight gain, survival rate and fish deformities were compared among four management regimes in each season: (1) fish larvae fed with live zooplankton (LF); (2) direct fertilization with poultry manure (PM); (3) direct fertilization with cow dung (CD); (4) a control system (C), where a commercial diet containing 32% crude protein was applied. The LF treatment produced significantly higher weight gain and survival rate of goldfish (P < 0.05) in both the trials through maintenance of better water quality and greater abundance of zooplankton in the system. Fish deformities were highest in the C treatment in both the experiments. Water temperature averaged 27.5ºC and 16.2ºC, respectively, in the summer and the winter trials. Average weight gain and survival rates of goldfish achieved during the winter trial were considerably lower than the summer trial (P < 0.05).

Key words*: aquaculture management, fish production, goldfish, seasonal effect, water quality.*

INTRODUCTION

The bulk of ornamental fishes in the international aquarium trade is of freshwater origin and is farm-raised (Livengood and Chapman, 2007). The goldfish, *Carassius auratus* (L.), is a very popular ornamental fish and has a market for individuals as small as 4 g (minimum), that typically requires only about ten weeks of growth to attain the saleable size (Jha et al., 2006a; Jha, 2017). One of the critical bottlenecks that culturists have to face is the survival of the larvae that has just made the transition from an endogenous to an exogenous feeding habit in nursery tank conditions. Now, the same larvae, which have grown to about two weeks, are stocked under intensive culture conditions for quick growth in short period. Therefore, the level of expertise required in production management, particularly with relation to water quality is higher with ornamental fish than any other type of aquaculture (Watson and Shireman, 1996).

The fish are subjected to different kinds of aquaculture management that varies from farm to farm. The use of organic manures in ornamental fish production has been documented (Jha et al., 2004; Jha and Barat, 2005a). However, using organic manure can result in negative environmental impact (Jha, 2007) and supply of exogenous live food can be an effective alternative (Jha and Barat, 2005b; Jha et al., 2006b; 2008; Jha, 2019). Fish are unable to perform de novo synthesis of carotenoids (Goodwin, 1984) and rely on costly dietary supply to achieve their natural pigmentation (Paripatananont et al., 1999), since the market value of ornamental fish increases with intensity of skin colouration (Nica et al., 2019). Since Indian farmers are generally unable to provide costly dietary supplements, they stress on the supply of live food instead. Taking advantage of the tropical climate, fish culturists in India have the opportunity to harvest multiple crops throughout the year (Jha et al., 2007) where pond water temperature falls below 20ºC for only three months in a year, i.e. mid-November to mid-February.

In the present experiment, two 11-week growth trials were conducted during two different seasons (summer and winter) to assess the seasonal influence on the growth performance of goldfish, *Carassius auratus* (L.) larvae in earthen ponds maintained under different production management regimes. The study also aimed to explore the possibility of use of exogenous plankton in promoting higher survival and growth of goldfish, compared to the traditional application of organic manure.

MATERIALS AND METHODS

Among the four prominent seasons distinguished throughout a year, we selected the summer and winter, for the two 11-week growth experiments: (1) 14 December' 12 – 01 March' 13 (winter); and (2) 14 April' 13 – 29 June' 13 (summer). Each seasonal trial was conducted in twelve outdoor earthen ponds (9.14 x 6.10 x 1.07 m; capacity: 59650 l each) in an ornamental fish farm (Rainbow ornamentals) in Jalpaiguri, India. Fish were cultured for 11 weeks according to one of the four treatments: (1) introduction of live zooplankton (live-food system or LF); (2) direct fertilization with poultry manure (PM); (3) direct fertilization with cow dung (CD); and (4) introduction of a commercial pelleted food (Tokyu Corp., Japan) into the ponds (control system or C). Three similar ponds were randomly assigned for each treatment.

About two weeks old goldfish larvae with an average initial weight of 0.10 ± 0.013 g (winter) and 0.12 ± 0.009 g (summer) were used in the growth experiments (n = 250 in each case). The larvae were stocked at 0.3 fish/l density, as optimized in an earlier experiment with koi carp, *Cyprinus carpio* L. (Jha and Barat, 2005c). The fish in the LF ponds were fed by transferring about 1000 l of plankton-rich water at a fixed hour (7 A.M.) every day in each pond from a series of plankton culture ponds that were fertilized with poultry manure and maintained under similar management conditions as the PM treatment. The entire experimental unit was covered by a single layer of bird netting. Constant water levels were maintained in the culture ponds by supplying ground water periodically to compensate for loss due to evaporation. Approximately 1000 l of excess water was discharged from each pond every day during the introduction of live plankton water.

The commercial diet applied in the control treatment was selected on the basis of widespread availability and contained 32% crude protein, 4% crude fat, 5% crude fibre, 10% crude ash, 9% moisture and 31% nitrogen free extract. The food was applied at 5% body weight of stocked fish once daily. The poultry and cow manures were collected from local farms, and allowed to decompose for 10 days prior to application. They were applied at a dose of 0.26 kg/m^3, every 10 days, in the PM and CD treatments, as standardized in an earlier experiment (Jha et al., 2004).

Samples of water were collected from each pond at a fixed hour of the day (9 A.M.) every week and were analyzed for various water quality parameters according to methods described in APHA (1998). Plankton samples were collected from all the ponds at about 5 hours after the introduction of plankton-rich water in the LF ponds with a plankton net made of standard bolting silk cloth (with 77 mesh/cm^2) two times a week. Collected plankton samples were concentrated to 20 ml and preserved in 4% formalin. Enumerations of 1 ml of concentrated plankton were performed under a stereoscopic microscope using a Sedgwick Rafter Counting Cell.

Fish were harvested after 11 weeks of each trial. 250 fish were randomly selected from each treatment and weighed individually to the nearest 0.01 g. For this the fish were anaesthetized with tricaine methane sulphonate (MS – 222) of 0.04 g/l concentration. The Specific Growth Rate (SGR) was calculated as: SGR = 100 [(ln W_t – ln W_0)/t]; where W_0 and W_t are the initial and final live weight of fish (g), respectively, and (t) is the culture period in days (Ricker, 1975). The number of fish with body deformities was also recorded during harvest. Dead fish were removed daily, they were not replaced during the course of study, and differences between the number of fish stocked and the number of fish at harvest were used to calculate percent mortality in each treatment. Final survival and deformities percentage were normalized using the arc sin angular transformation method (Mosteller and Youtz, 1961) before being subjected to further statistical analysis.

The results were statistically evaluated. Data on fish growth, survival and deformities in each seasonal trial was pooled for one way analysis of variance (ANOVA), and further subjected to Tukey's Test (Zar, 1999) to determine significant differences between the means. Statistical significance was accepted at P < 0.05 levels.

RESULTS AND DISCUSSIONS

Pond water temperature averaged 27.5ºC in summer and 16.2ºC in the winter trial. The variations of water temperature in the winter and summer growth experiments are shown in Figure 1. The results of the various water quality parameters in the experimental tanks during the different growth experiments are presented in Table 1. Values of dissolved oxygen (DO) and pH were significantly higher in LF and C (P < 0.05) than the manured treatments in both the seasonal experiments (Table 1).

Values of free CO_2, BOD, total alkalinity, phosphate, ammonium and nitrate were significantly higher (P < 0.05) in the manured treatments (PM and CD), compared to LF and C in both the experiments (Table 1). On an average, the plankton volume was highest in the PM treatment followed in decreasing order by the CD, LF and C treatments in both the experiments. However, the cladoceran population was highest in the LF treatment in both the cases (Figure 2).

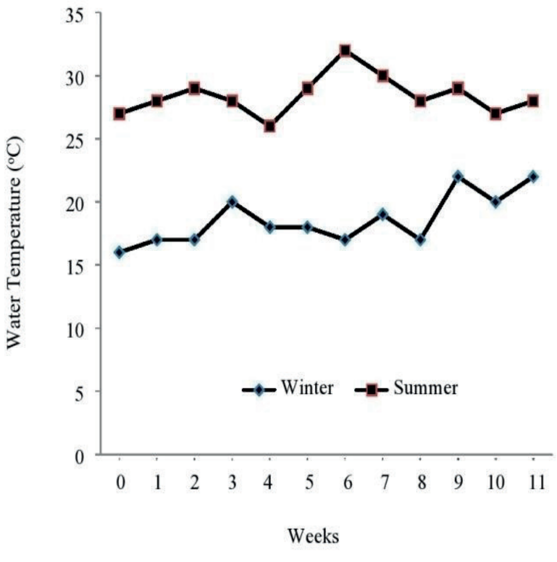

Figure 1. Weekly mean water temperature (ºC) recorded from the 12 fish culture ponds at 09.00 AM during the winter and summer growth experiments

In both the experiments, the average harvest weight of goldfish was highest (P < 0.05) in the LF treatment, followed in decreasing order by the PM, CD and C treatments (Table 2). Highest survival rates were obtained in the LF treatment (P < 0.05). The control treatment produced the highest number of deformed goldfish, while the lowest numbers were recorded in the LF treatment (P < 0.05). The average weight gain and survival rate of carp was lower during the winter growth trial in all the treatments, compared to the summer trial (Table 2).

In both the seasonal experiments, supply of exogenous plankton (LF) substantially improved weight gain (P < 0.05) and fish survival (P < 0.05), compared to the manured treatments (PM and CD) and control (C). Organic manures, if not decomposed completely before application in aquaculture pond may result in water quality degradation as they utilize oxygen during decomposition.

Therefore, the amount of any organic manure to be added in the pond mainly depends upon its biological oxygen demand, as their use may cause severe dissolved oxygen depletion in the pond and result in production of toxic gases, besides contributing to diseases (Chakrabarty et al., 2008).

Although the manures were allowed to decompose for 10 days prior to application in both the seasonal experiments, it could be possible that they were not properly decomposed and resulted in reduced water quality in the manured treatments.

Since the LF treatment received only the plankton rich water from the plankton culture ponds (maintained very similar to the PM treatment), it may well be that the toxic metabolites that were not assimilated by the phytoplankton and were the cause of water quality depletion in the manured ponds were severely diluted in the LF treatment and caused less damage.

Even the zooplankton abundance (no./l) was greater in LF, compared to PM and CD in both the seasonal experiments.

Table 1. Mean water quality parameters (SE in parentheses) in the four treatments recorded in the two growth trials during different seasons (winter and summer)

Culture period	Dissolved oxygen (mg/l)				pH (mg/l)			
	LF	PM	CD	C	LF	PM	CD	C
Winter	6.28^{a}	5.81^{b}	5.30^{c}	6.12^{ab}	7.25^{a}	6.26^{bc}	5.98^{c}	7.03^{ab}
	(0.20)	(0.25)	(0.15)	(0.10)	(0.12)	(0.23)	(0.15)	(0.15)
Summer	7.13^{a}	6.16^{c}	5.57^{d}	6.58^{b}	7.45^{a}	6.49^{b}	6.34^{b}	7.16^{ab}
	(0.29)	(0.20)	(0.21)	(0.19)	(0.18)	(0.22)	(0.28)	(0.20)
	Free CO_2 (mg/l)				BOD (mg/l)			
	LF	PM	CD	C	LF	PM	CD	C
Winter	2.21^{d}	3.32^{ab}	3.84^{a}	2.77^{bc}	0.91^{bc}	2.81^{a}	2.75^{a}	1.31^{b}
	(0.15)	(0.24)	(0.21)	(0.12)	(0.12)	(0.21)	(0.26)	(0.11)
Summer	1.42^{c}	2.49^{b}	3.17^{a}	2.19^{b}	0.78^{b}	1.96^{a}	1.90^{a}	1.01^{b}
	(0.12)	(0.15)	(0.22)	(0.20)	(0.07)	(0.30)	(0.18)	(0.11)
	NH_4-N (mg/l)				Alkalinity (mg/l)			
	LF	PM	CD	C	LF	PM	CD	C
Winter	0.09^{b}	0.34^{a}	0.38^{a}	0.13^{b}	35.70^{bc}	75.55^{a}	70.48^{a}	50.26^{b}
	(0.02)	(0.04)	(0.04)	(0.02)	(3.28)	(7.19)	(6.68)	(4.14)
Summer	0.10^{b}	0.28^{a}	0.30^{a}	0.12^{b}	31.26^{b}	70.85^{a}	65.55^{a}	38.25^{b}
	(0.01)	(0.03)	(0.04)	(0.02)	(2.86)	(6.66)	(6.14)	(5.36)
	NO_3-N (mg/l)				PO_4-P (mg/l)			
	LF	PM	CD	C	LF	PM	CD	C
Winter	0.05^{b}	0.29^{a}	0.24^{a}	0.08^{b}	0.12^{c}	0.62^{a}	0.55^{a}	0.26^{b}
	(0.01)	(0.03)	(0.02)	(0.01)	(0.01)	(0.08)	(0.04)	(0.02)
Summer	0.06^{b}	0.20^{a}	0.18^{a}	0.07^{b}	0.18^{b}	0.40^{a}	0.45^{a}	0.22^{b}
	(0.01)	(0.03)	(0.03)	(0.01)	(0.02)	(0.05)	(0.06)	(0.02)

Each mean value represents 12 samples collected at weekly intervals during each of the four 11-week seasonal trials. Different superscripts of each water quality parameter in the same row indicate statistically significant differences between means at 5% level. The treatments correspond to earthen ponds treated with live-food (LF), poultry manure (PM), cow dung (CD), and control (C)

Table 2. Summary of growth, SGR, deformities and survival rate of goldfish, *Carassius auratus* (L.), produced in the two growth experiments during different seasons (winter and summer)

	Treatment			
	LF	PM	CD	C
Harvest weight (g ± SE)				
Winter	4.35 ± 0.21^{a}	3.87 ± 0.11^{b}	3.56 ± 0.11^{bc}	2.25 ± 0.09^{d}
Summer	7.62 ± 0.27^{a}	5.23 ± 0.15^{b}	4.32 ± 0.14^{c}	3.50 ± 0.08^{d}
Weight gain (g ± SE)				
Winter	4.25 ± 0.21^{a}	3.77 ± 0.11^{b}	3.46 ± 0.11^{bc}	2.15 ± 0.09^{d}
Summer	7.50 ± 0.27^{a}	5.11 ± 0.15^{b}	4.20 ± 0.14^{c}	3.38 ± 0.08^{d}
SGR (%/ day)				
Winter	4.83 ± 0.21^{a}	4.68 ± 0.11^{b}	4.58 ± 0.11^{c}	3.99 ± 0.09^{d}
Summer	5.32 ± 0.27^{a}	4.83 ± 0.15^{b}	4.59 ± 0.14^{c}	4.32 ± 0.08^{d}
Deformed individuals (%)				
Winter	2.8^{d}	5.7^{c}	9.1^{b}	18.1^{a}
Summer	0.9^{d}	3.9^{c}	5.6^{b}	12.5^{a}
Survival rate (%)				
Winter	90.1^{a}	79.2^{b}	70.2^{c}	67.5^{c}
Summer	96.3^{a}	88.9^{b}	80.5^{c}	74.4^{d}

Each mean value (apart from survival rate) represents 250 randomly selected samples during harvest. Different superscripts in the same row indicate statistically differences between means at 5% level. Treatments represent earthen ponds treated with live-food (LF), poultry manure (PM), cow dung (CD), and control (C)

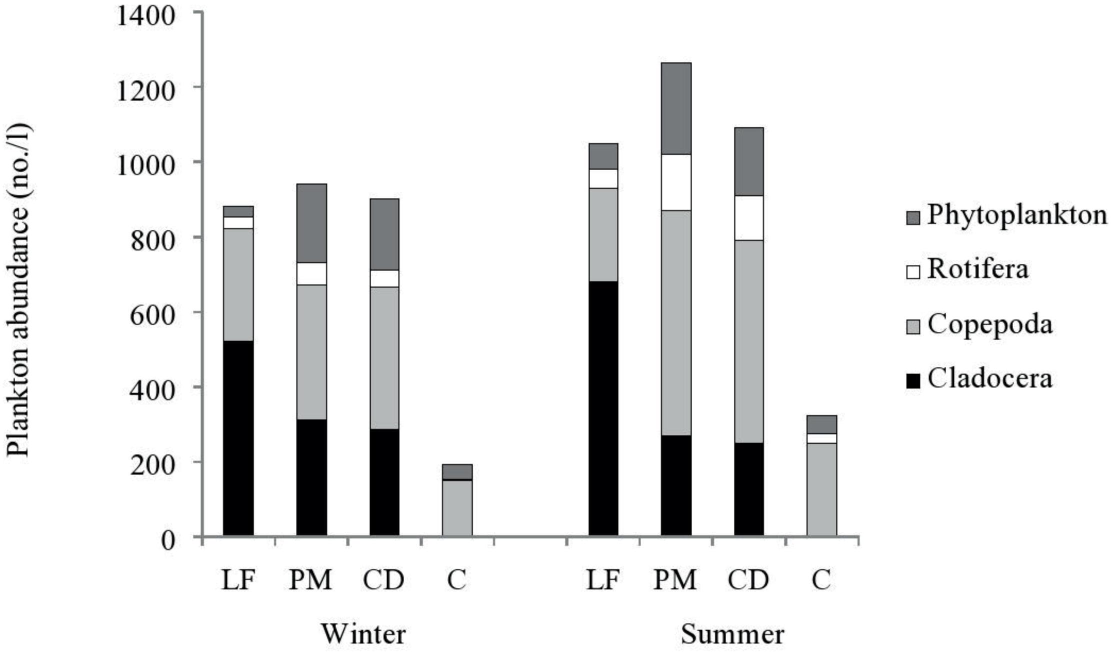

Figure 2. Plankton abundance (no./l) in the four treatments during the winter and summer growth experiments

Cladocerans are easily digestible due to the presence of digestive enzymes (Kumar et al., 2005) and they have high energetic caloric value (Morris and Mischke, 1999). The life history parameters of several cladocerans (Jana and Pal, 1985; Urabe and Watanabe, 1992) suggest a definitive role of the culture media in their growth and reproduction. Maximum concentration of cladocerans in the LF treatment could be a consequence of improved water quality and is in agreement with earlier findings (Jha and Barat, 2005b; Jha et al., 2006a; 2006b; 2008).

The zooplankton concentration in any particular treatment was higher in the summer experiment, compared to winter (Figure 2).

The phytoplankton and zooplankton communities, which develop in rearing ponds, are influenced by interactions between temperature, photoperiod, water quality, nutrient availability and fish predation (Geiger, 1983). The egg production and hatching rate of zooplankton are normally lower at low temperature, and generally increase with increasing temperature up to a thermal threshold (Santhanam et al., 2013). Similar trend has been reported by Longoria (2003), Rhyne et al. (2009), and Santhanam and Perumal (2012). On the other hand, Dodson (1974) had suggested that in summer, planktonic individuals have a reproductive disadvantage because of energetic costs associated with the development of the antipredator structures. Some researchers have shown that the chemicals released by invertebrate predators may induce the defensive morphologies, reduce growth rate, brood size, and survival rate of certain zooplankton (Havel and Dodson, 1987; Ketola and Vuorinen, 1989). However, in properly maintained aquaculture ponds as employed in the present experiments, there was reduced risk of invertebrate predator infestation and the zooplankton were primarily consumed by the cultured fish that was similar in both the seasonal trials. Lower weight gain of goldfish during the winter trial in our experiment may also be related to the lower abundance (no./l) of zooplankton in winter (Figure 2). Similar decrease in zooplankton abundance during winter was been reported by Dhawan and Kaur (2002), and Jha et al. (2006b).

Temperature is one of the most important factors determining somatic growth of fish (Hofmann and Fischer, 2003). The goldfish are known to tolerate extreme and fluctuating temperatures (Reynolds and Casterlin, 1979; Ford and Beitinger, 2005). Growth performance in a related species, the crucian carp (*Carassius carassius*) is also known to be influenced by temperature (Holopainen et al., 1997; Coop et al., 2010). For any fish species, there is no fixed temperature preference as such. Rather, in a large waterbody, a fish does not move to water of a given temperature within a gradient and then remain there, but tends to make 'exploratory movements' into waters of both lower and higher temperature (Jobling, 1981). Therefore, it may be more realistic to consider a temperature range rather than a fixed temperature. In our experiment, average water temperature during the winter growth experiment (range: 16°C-22°C; n = 12) was 11.3°C lower than the summer average (range: 26°C-32°C; n = 12). It appears that the temperature range of 26°C-32°C suited better growth and survival of goldfish, compared to lower temperatures. Survival rates of goldfish ranged from 68.5% (C) to 90.1% (LF) in the winter trial, and were lower by 6.2% to 10.3% in the different treatments, compared with the summer trial (Table 2). In our experiment, weight gain of goldfish in all the treatments was much lower in winter, compared with the summer growth experiment (Table 2). The results correspond to an earlier experiment with koi carp where an 83.8% increase in weight gain was achieved during the summer, compared with a winter growth experiment (Jha et al., 2007). According to Horvath et al. (1992), metabolism and food demand of carp decreases gradually with decreasing water temperatures below 20°C, leading to lower growth rates. Information relating to the growth of estuary grouper, *Epinephelus salmoides* from the experimental cage sites showed that the annual temperature conditions (28-32°C) were close to those promoting maximum growth (Chua and Teng, 1980; Jobling, 1981).

Significantly higher ($P < 0.05$) incidences of fish deformities were obtained in the control treatment in both the seasonal experiments. This could be attributed to nutritional deficiencies caused by lower abundance of plankton and possible leaching of nutrients out of the pelleted food (Jha, 2007; 2010; Myszkowski et al., 2002). In ornamental high-value species such as goldfish (*Carassius auratus*), emphasis should be given to achieving high levels of skin pigmentation, body shape, fin shape and body size, that are the most important quality criteria informing their market value (Paripatananont et al., 1999). The percentage of fish deformities were higher in the winter trial, compared to summer.

CONCLUSIONS

From the present investigation, the winter season appeared to be less productive for goldfish culture, compared to summer. In both the seasonal experiments, the live-food treatment (LF) appeared to be the most effective for goldfish culture compared to poultry manure (PM) or cow dung (CD) treatments, through maintenance of better water quality and greater abundance of zooplankton in the system.

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EFFECTS OF DIFFERENT PLANT GROWTH REGULATORS IN A VEGETATIVE CHAMBER ON SEEDLING MORPHOLOGY PARAMETERS OF YELLOW MUSTARD (*Brassica juncea* L.)

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Abstract

The aim of this study was to evaluate the sensitivity of yellow mustard (Brassica juncea L) to plant regulators compound growth by the root length, root surface area, root volume, stem length and surface area. The experiment consists of three varieties of yellow mustard (RETRO, FELICIA and PRIMA) and eight growth regulators (Albit, Vermistimd, Antistress, Agrios, Regoplan, Biofoge, Stimulate, Fast start). The results showed that the growth compound regulator was the main factor causing the difference in root length and had no correlation with the variety. Different types of plant growth compound regulators have significant differences in the growth and development of mustard. For RETRO, the growth compound regulator VERMISTIMD significantly increased root growth compared with the control. In FELICIA, the length of root has changed under the influence of seeds inoculation with growth compound regulator BIOFOGE. However, the treatment of ANTISTRESS significantly inhibited the root length of FELICIA and RETRO. In the study of the PRIMA, all the growth regulators had no significant or inhibitory effect on the growth of the roots. In addition, there were also differences in the sensitivity of compound growth regulators to root and stem, for stem growth of FELICIA, the growth compound regulator VERMISTIMD has the opposite effect on the stem surface area and BIOFOGE has a significant impact on stem surface area of PRIMA.

***Key words**: mustard, plant growth regulator, culture room, morphology parameters.*

INTRODUCTION

Mustard is an important cash crop. It is one of the world's major sources of vegetable oil and vegetable protein. Mustard seed oil is famous for its rich content in isothiocyanates, which has been proved to have a key role in cancer prevention and bactericidal, and it has attracted more and more attention (Delaquis and Mazza, 1995; Trachootham et al., 2006; Melnik et al., 2015). Many studies have shown that mustard oil is considered one of the healthiest cooking oils, which is widely used for food, as well as in many industries - canning, bakery, confectionery, margarine, soap and pharmaceutical (Shekhawat et al., 2012; Zhuikov, 2014).

On the other hand, Mustard is favour by its appetizing flavor and preservative value (Bin Mustafa et al., 2018). The vigor of the plant at the beginning of germination has an important role for the whole growth period, especially root vitality. Previous studies have shown that increasing root length and increasing root volume are conducive to the accumulation of dry matter, and the surface area of roots is related to the rate of nutrient uptake (Ali et al., 2011). The plasticity of the root system is generally regulated by the availability of specific nutrients (López-Bucio, et al., 2003). In individual plants, the growth and development of roots is also affected by both the shoot and the above-ground environment.

As an exogenous stimulant, growth regulators have a prominent effect on promoting root growth, increasing crop yield components and quality formation (Wu et al., 2012; Setia et al. 1989). Furthermore, growth regulators play important roles in mitigating the effects of abiotic adversity on crops involved in the regulation the number and vitality of root systems (Ali et al., 2011; Yuan et al., 2014). Silva-Matos et al. demonstrated that foliar spray of humic substances enhances aerial part and root system of watermelon seedlings (Silva-Matos et al., 2019).

Maize treated with exogenous spermidine increased leaf area to enhance photosynthesis, promoted root growth to improve water absorption, and finally increased maize yield (Li, 2019). Wang Tao reported that inoculating DJ515-2 fungal could improve the seed vitality of mustard and seed germination rate, root length, stem length and seed vitality index of the inoculated group increased by 20.0%, 52.4%, 56.3% and 83.7%, respectively compared with the control group (Wang et al., 2018). However, the use of single component regulators is generally targeted to conditions and crop varieties, not broad spectrum, limited yield increase, so the broad spectrum of compound regulators are strongly required in production. Liu Yali found that the mix of two or more plant growth regulators produces better results than a single application when applied to wheat leaf surfaces (Liu et al., 2005). Seed mixing with compound regulator can significantly increase the content of ZR and GA and the activity of catalase in leaves of progeria maize varieties, and reduce the content of ABA, H_2O_2 and MDA, thus delaying the progeria of leaves (Shao et al., 2014). Application of plant growth compound regulator can affect stress resistance and yield by adjusting plant height (Han et al., 2016).

Previous studies have reported the use of growth regulators to treat mustard seeds before germination can improve germination rate, uniform seedling emergence, strong stress resistance (Dalyan et al., 2018; Asgher et al., 2018; Sharma et al., 2016), but different genotypes of mustard have different responses to different plant growth compound regulators. Therefore, the objective of the study was to determine the sensitivity of mustard to different growth compound regulators in comparison with the growth and development of plants. Moreover, it is important guiding significance for the use of growth compound regulators in the field.

MATERIALS AND METHODS

Seeds of yellow mustard (*Brassica juncea* L.) cultivars RETRO, FELICIA and PRIMA. These cultivars were selected based on their yield potential, flowering/maturity groups, seed availability and popularity among Ukrainian growers during the period of these experiments. The seeds were procured from certified (commercial and/or Govt.) sources.

This experiment is the result of research cooperation between Henan Institute of Science and Technology (China) and Sumy National Agrarian University (Ukraine).

The experiment was carried out during the 2018 and 2019 years at the laboratories of Henan Institute of Science and Technology.

Eight compound regulators were used in the experiment, Albit, Vermistimd, Antistress, Agrinos, Regoplan, Biofoge, Stimulate, Fast Start, respectively. These seeds were soaked with either deionized water or different growth regulators. In all experiments seeds were germinated in plastic Petri dishes 90 mm square containing two germination papers saturated with Nutrient solution. All experiments were carried out in a culture room under a regime of 13 h light 11 h darkness, with temperatures of (30 ± 2) °C during the day and (25 ± 2)°C at night. There were 50 seeds per dish and the treatment had three repetitions. The composition of the nutrient solution is: 2.5 $mmol \cdot l^{-1}$ $Ca(NO3)_2$, 1 $mmol \cdot l^{-1}$ $MgSO_4$, 0.5 $mmol \cdot l^{-1}$ $(NH_4)H_2PO_4$, 2.5 $mmol \cdot l^{-1}$ KCl, 2 $mmol \cdot l^{-1}$NaCl, 2×10^{-4} $mmol \cdot l^{-1}$ $CuSO_4$, 1×10^{-3} $mmol \cdot l^{-1}$ $ZnSO_4$, 0.1 $mmol \cdot l^{-1}$ EDTA FeNa, 2×10^{-2} $mmol \cdot l^{-1}$ H_3BO_3, 5×10^{-6} $mmol \cdot l^{-1}$ $(NH4)_6Mo_7O_{24}$, and 1×10^{-3} $mmol \cdot l^{-1}$ $MnSO_4$. After 3 days of culture, plant samples were taken for measurement.

Measurement of morphological parameters

Mustard seedlings were separated into root and stem sections. Primary root length (PRL) was defined as the distance from the junction to the root apex and plant height defined as the distance from the junction to the shoot apex. The whole root was scanned using an Epson scanner (Seiko Epson Corp., Tokyo, Japan). The total root length (TRL) was calculated using WinRHIZO Version 4.0 b (Regent Instruments Inc., Quebec, Canada). Stems were scanned, then calculated using WinRHIZO.

Statistical analysis

Analysis of morphological parameters was based on five plants from three replicates of each treatment. All data were analyzed by ANOVA and Duncan's multiple range tests, and differences among compared means were deemed significant if $P < 0.05$. Data are expressed as mean ± standard error.

RESULTS AND DISCUSSIONS

Correlation analysis of variety and growth compound regulators on root length

There are many factors influencing root growth, and the correlation between varieties and growth regulators on root length was found the factors affecting root length are mainly derived from growth compound regulators, and the significance is 0 ($P < 0.01$), which has no relationship with the variety.

It is indicated that growth regulators are the main factors affecting root length (Table 1).

Table 1. Effect of growth compound regulators on root morphology of mustard seedlings in a vegetative chamber

Variety (Factor A)	Growth regulator (Factor B)	Root length cm	Root volume cm^3	Root surf area cm^2
RETRO	CK	3.94±0.61b	0.01±0.01abc	0.48±0.07bc
	ALBIT	3.89±0.6b	0±0c	0.44±0.05bc
	VERMISTIMD	4.76±0.7a	0.01±0a	0.58±0.06a
	ANTISTRESS	1.97±0.29d	0±0c	0.3±0.04d
	AGRINOS	3.96±0.36b	0.01±0.01abc	0.52±0.13ab
	REGOPLAN	3.22±0.3c	0.01±0.01ab	0.46±0.06bc
	BIOFOGE	3.58±0.48bc	0±0bc	0.41±0.05c
	STIMULATE	3.19±0.57c	0.01±0.01ab	0.47±0.09bc
	FAST START	3.34±0.31bc	0±0.01abc	0.41±0.1c
FELICIA	CK	3.81±0.55ab	0.01±0b	0.54±0.03ab
	ALBIT	3.58±0.51bc	0±0b	0.45±0.07b
	VERMISTIMD	4.31±0.62ab	0.01±0b	0.54±0.07ab
	ANTISTRESS	2.56±0.41d	0.01±0b	0.4±0.07b
	AGRINOS	3.82±0.58ab	0±0b	0.46±0.13b
	REGOPLAN	4.24±1.75ab	0.01±0.01a	0.73±0.47a
	BIOFOGE	4.65±1a	0.01±0b	0.58±0.08ab
	STIMULATE	2.72±0.27cd	0.01±0b	0.46±0.06b
	FAST START	3.87±0.43ab	0±0b	0.45±0.09b
PRIMA	CK	5.24±0.82a	0.01±0.01ab	0.56±0.09a
	ALBIT	3.88±1.04bc	0±0b	0.42±0.12bc
	VERMISTIMD	3.44±0.82c	0±0b	0.4±0.05c
	ANTISTRESS	3.58±0.91bc	0.01±0a	0.56±0.13a
	AGRINOS	4.57±0.81ab	0±0.01ab	0.53±0.1ab
	REGOPLAN	2.93±0.66c	0.01±0a	0.51±0.12abc
	BIOFOGE	3.37±0.5c	0±0b	0.4±0.06bc
	STIMULATE	3.13±0.74c	0.01±0a	0.48±0.08abc
	FAST START	3.5±1.17bc	0.01±0a	0.49±0.11abc

Note: Different letters in the same column indicate the significant level at 5%.

Effect of growth compound regulators on root morphology of mustard seedlings

Compared with the control, different types of plant growth compound regulators have significant differences in the growth and development of mustard roots (Table 1). For RETRO, the growth regulator VERMISTIMD significantly increased root length, root volume and root surface area according to observation, particularly in terms of the root elongation. The root length is 4.76 cm with treatment of VERMISTIMD, which is 0.82 longer than 3.94 cm in control. However, the root length combined treatment of ANTISTRESS was only 1.9 cm, which markedly inhibited the growth of the roots. In FELICIA, the length of root has changed under the influence of seeds inoculation with growth regulator BIOFOGE on 4.65 cm, while only 3.81 cm in control.

There was no significant change in root volume and root surface area with the inoculation of ANTISTRESS, but root length significantly ($p < 0.05$) reduced with the ANTISTRESS application by 2.56 cm. In the study of the PRIMA, all the growth regulators had no significant or inhibitory effect on the growth of the roots. The root length and root surface area were the largest without the growth regulator, which were 5.24 cm and 0.56 cm^2, respectively. Considering the root parameter, different varieties have different sensitivities to different growth regulators.

Effect of growth compound regulators on stem morphology of mustard seedlings

The results of the stem of the seedlings vary with growth compound regulators (Table 2).

Table 2. Effect of growth compound regulators on stem morphology of mustard seedlings in a vegetative chamber

Variety (Factor A)	Growth regulator (Factor B)	Stem length, cm	Stem surf area, cm^2
RETRO	CK	1.67±0.39a	0.45±0.09a
	ALBIT	1.42±0.26a	0.34±0.05ab
	VERMISTIMD	1.23±1.15a	0.35±0.22ab
	ANTISTRESS	1.19±0.21a	0.28±0.05b
	AGRINOS	1.57±0.42a	0.39±0.09ab
	REGOPLAN	1.66±0.21a	0.38±0.06ab
	BIOFOGE	1.73±0.26a	0.42±0.05a
	STIMULATE	1.54±0.24a	0.38±0.06ab
	FAST START	1.57±0.31a	0.37±0.08ab
FELICIA	CK	1.83±0.29ab	0.49±0.08a
	ALBIT	1.64±0.15b	0.38±0.05bc
	VERMISTIMD	1.4±0.43b	0.34±0.11c
	ANTISTRESS	1.52±0.21b	0.42±0.05abc
	AGRINOS	1.66±0.13b	0.42±0.04abc
	REGOPLAN	2.24±0.95a	0.42±0.09abc
	BIOFOGE	1.85±0.17ab	0.47±0.06ab
	STIMULATE	1.38±0.14b	0.33±0.01c
	FAST START	1.72±0.25b	0.4±0.07bc
PRIMA	CK	1.55±0.27ab	0.44±0.06a
	ALBIT	1.78±0.42a	0.43±0.09a
	VERMISTIMD	1.81±0.15a	0.43±0.06a
	ANTISTRESS	1.49±0.09ab	0.38±0.03ab
	AGRINOS	1.49±0.34ab	0.38±0.07ab
	REGOPLAN	1.48±0.57ab	0.31±0.08bc
	BIOFOGE	1.16±0.59b	0.27±0.1c
	STIMULATE	1.69±0.16a	0.4±0.03a
	FAST START	1.56±0.47ab	0.35±0.08abc

Note: Different letters in the same column indicate the significant level at 5%.

Stem length and surface area were no significant difference in the sensitivity of RETRO to different growth regulators compared to control. In FELICIA, the growth regulators of REGOPLAN and BIOFOGE slightly promoted the length of the stems, 2.24 cm and 1.85 cm, respectively, but the effect was not significant, while VERMISTIMD and STIMULATE have a significant inhibitory effect on the stem surface area. Seed pre-soaking with VERMISTIMD, ALBIT and STIMULATE increased the stem length and stem surface area for PRIMA, and BIOFOGE has a significant impact on stem surface area.

CONCLUSIONS

Plant growth compound regulators control most of the characteristics of growth system. Zhang Zigao reported that the morphology of wheat seedlings treated with complex excitin was improved (Zhang et al., 1993). HAN Yiqiang pointed out application of plant growth regulators B (main ingredients: ethephon 160 mg/l, 2-N.N-diethylaminocaproate 20 mg/l) obviously shortened the distance between the first node to forth node, then decreased the plant height, and shortened growth period duration, then insured the mature of 'Nongda 108'and increased maize yield (Han et al., 2016). The eight compound growth regulators used in this experiment can be divided into three categories: one is to promote seedling development, one is to inhibit effect, and the third is to have no effect. For example, the growth compound regulator VERMISTIMD significantly promoted root growth of RETRO according to data, particularly in terms of the root elongation, and growth compound regulator BIOFOGE significantly increased the root length of FELICIA, and the growth compound regulators of REGOPLAN and BIOFOGE slightly promoted the stem length. The pre-soaking with growth compound regulators VERMISTIMD, ALBIT and STIMULATE increased the stem length and stem surface area for PRIMA. On the other hand, root length significantly reduced with the ANTISTRESS application. The results also showed that there were differences in the sensitivity of root and stem to growth regulators, such as, the growth compound regulator VERMISTIMD has the opposite effect on the stem surface area of FELICIA The different compositions of the eight growth compound regulators used in this experiment may be the main reason for the different experimental results. Further studies will be conducted on the effects of the components of the compound growth regulator on plant development and the interactions between the components.

According to the results, the sensitivity of mustard to plant growth compound regulators varies widely. The growth compound regulator VERMISTIMD had a significant effect on root growth of RETRO and FELICIA, but had no significant or inhibitory effect on the roots growth of PRIMA variety. This may be due to differences in growth regulator composition and variety specificity.

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ANTIOXIDANT POTENTIAL AND SOME MINERAL CONTENTS OF WILD EDIBLE MUSHROOM *Ramaria stricta*

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Abstract

People have used mushrooms for many different diseases for thousands of years. Although mushrooms are considered as nutrients for the first time, it has been determined by researches that they have medicinal properties. In this study, antioxidant status, oxidant status and some mineral contents of wild edible mushroom Ramaria stricta (Pers.) Quél were determined. The antioxidant and oxidant states of the mushroom were measured using Rel Assay kits. Element contents were determined using atomic absorption spectrometry. In this study, the antioxidant potential of R. stricta mushroom used in our study has been evaluated to be high in term of total antioxidant status (TAS) values (4.223 ± 0.054 mmol/l), total oxidant status (TOS) values (8.201 ± 0.095 µmol/l), and oxidative stress index (OSI) value (0.194 ± 0.001). Fruitbodies of R. stricta contains essential mineral elements which are of immense health benefit. The highest Fe content (451.21 ± 5.56 mg.kg^{-1}) was found in mushroom samples. It was established high Cu content (95.54 ± 2.06 mg.kg^{-1}), and Zn content (39.19 ± 1.07 mg.kg^{-1}). Ni content was 7.17 ± 0.32 mg.kg^{-1}. A lower content (2.18 ± 0.10 mg.kg^{-1}) was recorded for Pb. In addition, element contents were found to be at normal levels. As a result, R. stricta mushroom is thought to be a natural antioxidant source.

Key words*: antioxidant, oxidant, edible mushroom, Ramaria stricta.*

INTRODUCTION

Mushrooms are one of the most important elements of natural ecosystems. They are the indispensable elements of the ecological cycle. They are involved in the disintegration of organic matter in environments where they spread (Canli et al., 2016; İnci and Kırbag, 2018). Besides their ecological importance, mushrooms are important natural foods. It is one of the delicious foods of the human diet. They are rich in minerals (Gürgen et al., 2018). They also contain many essential amino acids, proteins, being rich in vitamin B. Edible mushrooms proved significant medicinal properties due to their high polysaccharide content, especially β-glucans (İnci et al., 2019).
Many studies have shown that mushrooms have different medicinal properties (Gedik et al., 2019). In previous studies, mushrooms have been reported to have many different activities such as antioxidant, antimicrobial, anti-cancer, anti-angiogenic, anti-inflammatory, anti-proliferative, antitumor, anti-HIV, anti-genotoxic, anti-aging, anti-gout, anti-cholinesterase, anti-hyperlipemia, anti-gastric ulcer activity, anti-allergy, DNA protective activity, anti-hypoxic, anti-diabetic, Immunomodulatory activity and anti-thrombotic activity (de Oliveira et al., 2002; Jose et al., 2002; Song et al., 2003; Kim et al., 2004; Lavi et al., 2006; Bae et al., 2007; El Dine et al., 2008; Weng et al., 2010; Kang et al., 2011; Kim et al., 2013; Li et al., 2015; Pandimeena et al., 2015; Devi and Maiti, 2016; Nguyen et al., 2016; Bal et al., 2017; Sevindik et al., 2018a).
Ramaria is a genus with about 200 species. There are species that spread in different ecosystems of the world of *Ramaria* genus. In addition to *R. formosa* and *R. pallida*, which cause nausea, vomiting and diarrhea, there are also types such as edible *R. flava*. The antioxidant activities of some *Ramaria* species are shown in Table 1. *Ramaria stricta* (Pers.) Quél., commonly known as the strict-branch coral of the genus *Ramaria* (Petersen and Scates, 1988; Braeuer et al., 2018). It has a

cosmopolitan distribution, and grows on dead wood, stumps, trunks, and branches of both deciduous and coniferous trees (Kuo, 2009).

Table 1. Antioxidant activities of *Ramaria* species

Mushroom species	Country	Extraction	References
Ramaria flava	China, Turkey	Ethanol, Hexane, Methanol, Aqueous	Gursoy et al., 2010; Liu et al., 2013; Öztürk et al., 2014; Bozdogan et al., 2016; Sadi et al., 2016
R. largentii	Romania	Ethanol	Aprotosoaie et al., 2017
R. botrytis	China	Water, Methanol, petroleum ether	Kim and Li, 2003; Froufe et al., 2009; Li 2017
R. aurea	India	Ethanol, methanol, Aqueous	Khatua et al., 2015; Zengin et al., 2017
R. formosa	India	Methanol	Ramesh and Pattar, 2010
R. botrytoides	China	Methanol	Guo et al., 2012
R. subalpina	India	Methanol	Acharya et al., 2017
R. fennica	Turkey	Methanol	Bakır et al., 2018

The aim of this study is the determination of antioxidant status, oxidant status and Fe, Zn, Cu, Pb and Ni contents of wild edible mushroom *Ramaria stricta* (Pers.) Quél.

MATERIALS AND METHODS

R. stricta samples were collected in 2019 from Bilyayivka, Odessa region (Ukraine). The mushroom samples were dried in an incubator at 40°C. 30 g of dried mushroom samples were weighed and powdered. Then, mushroom was extracted with ethanol (EtOH) in the Soxleth apparatus during approximately 6 hours at 50°C. A rotary evaporator was used to remove the solvent from the obtained extract. The extracts were kept at +4°C until experiment.

Total Antioxidant and Oxidant Studies

Total antioxidant status (TAS) values were determined using Rel Assay TAS kits (Erel, 2004). Total oxidant status (TOS) values were determined using Rel Assay TOS kits (Erel, 2005). Trolox was used as TAS calibrator and hydrogen peroxide was used as TOS calibrator. Oxidative stress index (OSI) value (Arbitrary unit: AU) was determined according to the formula below (Erel, 2005):

$$\text{OSI (AU)} = \frac{\text{TOS, µmol } H_2O_2 \text{ equiv./l}}{\text{TAS, mmol Trolox equiv./l X 10}}$$

Determination of Element Content

Samples were dried at 80ºC until constant weight to determine the Fe, Zn, Cu, Pb, and Ni contents of *R. stricta*. It was mineralized in a mixture of 9 ml HNO_3+1 ml H_2O_2 in the microwave solubilizer (Milestone Ethos Easy) by taking 0.5 g of 5 samples. The element contents of the samples were determined using an atomic absorption spectrophotometer device (Agilent 240FS AA) (Sevindik et al., 2017).

Statistical analysis

The experimental results were expressed as means ± SEM (standard error of the mean) of triplicates. The data was analysed with Excel statistical functions using the Microsoft Office 2016. Differences at $P \leq 0.05$ were considered to be significant.

RESULTS AND DISCUSSIONS

Total Antioxidant and Oxidant Status

Mushrooms have the potential to produce many antioxidant enzymes and coenzymes in secondary metabolite structure. Mushrooms, which are rich in nutrient content, contain vitamins with strong antioxidant characteristics such as A, C and E. In this context, it is very important natural products (Rathore et al., 2017). TAS values are an indicator of the endogenous antioxidants they produce (Selamoglu et al., 2016). Mushrooms with high TAS values have the potential to be an important antioxidant natural source. TOS values are an indicator of produced oxidant compounds (Selamoglu et al., 2017). High TOS values show that mushrooms produce more reactive oxygen types under the influence of environmental factors and people should pay attention to the consumption of these products. Thus, these indicators allow us to evaluate the antioxidant potential in the complex. However, there are very few studies to determine TAS, TOS and OSI values of mushrooms. In our study, TAS, TOS and OSI values of EtOH extract of *R. stricta* were determined. The findings obtained are shown in Table 2.

In literature, TAS, TOS and OSI values of *R. stricta* have not been determined. But, there are analogical studies of the antioxidant potential of different wild mushrooms.

Table 2. TAS, TOS and OSI values of *R. stricta*

Sample	TAS (mmol/l)	TOS (μmol/l)	OSI
R. stricta	4.223±0.054	8.201±0.095	0.194±0.001

*Values are presented as mean ± S.D
*Experiments were made as 5 parallel

Compared to these studies, TAS value of *R. stricta* was found higher than *Cerioporus varius* (2.312 mmol/l) (Sevindik, 2019), *Laetiporus sulphureus* (2.195 mmol/L) (Sevindik et al., 2018b), *Lepista nuda* (3.102 mmol/l) (Bal et al., 2019), *Lentinus tigrinus* (1.748 mmol/l) (Sevindik, 2018), and lower than *Leucoagaricus leucothites* (8.291 mmol/l) (Sevindik et al., 2018c).It is thought that this difference between the TAS values of mushrooms is due to the differences in their potential to produce antioxidant compounds. In addition, these differences may occur depending on the differences, levels, and diversity of secondary metabolites produced by the mushrooms as a reaction of the defense system depending on internal and external factors. The high TAS value of *R. stricta* in our study shows that mushrooms can be used as an important natural antioxidant source. In the literature, it was also reported that petroleum ether extracts of *R. stricta* show significant antioxidant activity (Sharma and Gautam, 2017).

Compared to TOS values of other mushroom species, it was established that TOS value of *R. stricta* was higher than those of *L. sulphureus* (1.303 μmol/l) (Sevindik et al., 2018b) and lower than *L. tigrinus* (19.294 μmol/l) (Sevindik, 2018), *C. varius* (14.358 μmol/l) (Sevindik, 2019), *L. nuda* (36.920 μmol/l) (Bal et al., 2019), and *L. leucothites* (10.797 μmol/l) (Sevindik et al., 2018c).

The differences in the TOS studies conducted with different mushroom species obtained from different localities attract attention.

It is thought that the main reason for this difference in TOS values is due to differences in the environmental factors and metabolic processes of the mushrooms. Consumption of a natural product with a high TOS value may pose problems for human health (Korkmaz et al., 2018).

And according to our results, the OSI values of *R. stricta* samples had higher values than *L. leucothites* (0.130) (Sevindik et al., 2018c) and *L. sulphureus* (0.059) (Sevindik et al., 2018b), and lower than *L. tigrinus* (1.106) (Sevindik, 2018), *C. varius* (0.627) (Sevindik, 2019), and *L. nuda* (1.190) (Bal et al., 2019). These differences in OSI values arise from the fact that the antioxidant system of mushroom is effective at different levels against oxidant compounds. As a result, OSI values of *R. stricta* were low.

This is because the fungi antioxidant system is potent and effective against oxidant compounds.

Element Contents

Mushrooms, which acts as a decomposing role in the ecosystem, accumulate different levels of elements within the body depending on the substrate content. Because of these properties, they are important element indicators (Baba et al., 2012). In this study, Fe, Cu, Zn, Pb and Ni contents of *R. stricta* were determined. The results are shown in Table 3.

Element contents may vary depending on the habitats collected from mushrooms consumed in different parts of the world. The maximum and minimum ranges of Fe, Zn, Cu, Pb and Ni levels detected in different wild mushrooms were shown in Table 4.

It was observed that the contents of Fe, Zn, Cu and Pb of *R. stricta* used in our study were in line with literature data. Ni content of *R. stricta* was found to be higher than the ranges specified in the literature.

Table 3. Element contents of *R. stricta*

Sample	**Fe** ($mg.kg^{-1}$)	**Zn** ($mg.kg^{-1}$)	**Cu** ($mg.kg^{-1}$)	**Pb** ($mg.kg^{-1}$)	**Ni** ($mg.kg^{-1}$)
R. stricta	451.21±5.56	39.19±1.07	95.54±2.06	2.18±0.10	7.17±0.32

Values are presented as mean ± S.D.
n = 3 (Experiments were made as 3 parallel).

Table 4. Range of reported literature values (mg/kg dry weight) of different mushrooms

Elements	Values (mg/kg dry weight)	References
Iron (Fe)	14.60-1714	Sarikurkcu et al., 2011; Sevindik et al., 2018a
Zinc (Zn)	7.63-240	Falandysz et al., 2007
Copper (Cu)	1.90-180	Falandysz et al., 2008; Sevindik et al., 2018c
Lead (Pb)	0.18-16.54	Nikkarinena and Mertanen, 2004; Sevindik et al., 2018a
Nickel (Ni)	0.67-6.72	Vetter, 1990; Sevindik et al., 2018a

CONCLUSIONS

In this study, total antioxidant level, total oxidant level, oxidative stress index and element contents of wild edible mushroom *R. stricta* were determined. Wild edible mushroom *R. stricta* is thought to be an important antioxidant source. It is recommended that daily consumption is limited in terms of element toxicity.

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EFFECT OF IRRIGATION AND FERTILIZATION ON THE CONTENT AND COMPOSITION OF HUMUS OF CHERNOZEM IN THE VEGETABLE-FODDER CROP ROTATION

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Abstract

Study was conducted in long-term field experiment (47 years) in irrigated vegetables cropping system on chernozem heavy loamy in Forest-Steppe zone of Ukraine. Different types of fertilization regimes were compared: mineral (NPK); organic (manure) and organo-mineral (manure + NPK). Humus content decreased by 10-12% after two 9-crops rotations compared to initial data (before irrigation) on all variants of experiment. Since the end of third 9 years cropping system humus has been increased up to 4.35% at the end of fifth crop rotation in organo-mineral fertilization regime. Mineral fertilization systems did not increase humus content compared to the control (without any fertilization). The highest humic compounds content in chernozem was observed on organic fertilization system. Long-term regular manure application led to significant increase of the content of humic complexes with calcium in comparison with mineral and organo-mineral fertilization regimes. Fulvic acids content was higher on variant with NPK application.

***Key words**: chernozem, composition of humus, different types of fertilization.*

INTRODUCTION

Climate change adaptation, mitigation and food security may be addressed at the same time by enhancing soil organic carbon sequestration through environmentally sound land management practices (Rumpel et al., 2020). Soils are the largest terrestrial reservoir of organic carbon (C) and thus play a significant role in the global C cycle. The loss of organic matter from agricultural lands constrains our ability to sustainably feed a growing population and mitigate the impacts of climate change (Machmuller et al., 2015). Also soil organic matter (SOM) has traditionally been viewed as the main yield determining factor in agriculture. Addressing these challenges development and implementation of practices that accumulate soil C are extremely important. Increase in agricultural production and productivity depends, to a large extent, on the availability of water. In arid and semi-arid regions, irrigation improves economic returns, but on the other hand irrigation can produce unwanted environmental consequences (Cirelli et al., 2009).

A meta-analysis (Zhou et al., 2016) showed that, on average across all biomes, drought and irrigation similarly induced minor increases in soil C pool by 1.45% and 1.27%, respectively. However, drought and irrigation oppositely affected both C fluxes and plant C pools as well as in agroecosystems (e.g., croplands and grasslands).

Active management of agricultural soils may reduce the losses of soil organic matter, but full life cycle analyses for fertilized and irrigated soils seldom show net carbon sequestration (McGill et al., 2018). Positive effects of irrigation on soil organic carbon become less pronounced at higher initial soil organic carbon contents and higher precipitation (Trost et al., 2013). Soils with higher initial carbon contents and higher soil moisture offer better living conditions for microorganisms. Inputs of carbon by the cultivation of crops exceed the microbial decomposition. In addition to increased input of carbon by improved plant growth, irrigation shows direct effects on soil aggregate building and thus on the ability of soil to fix organic carbon long-term (Trost et al., 2013).

Many researchers, as a general trend, note a slight decrease in humus stocks in the initial periods of irrigation and their gradual restoration over time, as well as a decrease of its content in the arable layer and an increase with depth (Komissarov, 2015).

The reduced soil organic carbon (SOC) content in irrigation treatment was attributed to drainage associated with greater stocking, together with accelerated decomposition of organic C resulting from elevated soil moisture maintained throughout the growing season (Condron et al., 2014).

During the growing season, there was a fluctuation in the humus content in the layer of 0-40 cm with an increase at the end of the irrigation season from 7 to 23% and a subsequent decrease of 9-10% compared to the initial values at the beginning of the next irrigation season (Voevodina, 2017).

Compared to non-irrigated areas, by irrigation with fresh water, the humus content is less than by irrigation with weakly mineralized water (Shedrin et al., 2017).

The combination of irrigation with other agronomic management factors also influences the development of soil organic carbon content. Authors (Stoner et al., 2019) observed that frequent irrigation decreases the amount of long-term stable C in pastures. Despite no difference in soil C accumulation, fertilized pastures store C longer than unfertilized pastures.

The application of organic fertilizers has an important influence on the carbon exchange of agro-ecosystems, especially in irrigated agriculture. The joint application of organic fertilizers in irrigated areas can maintain a high crop yield and increase the soil organic carbon content and CO_2 net absorption of paddy soil ecosystems (Shihong et al., 2018).

MATERIALS AND METHODS

Long-term field experiment was conducted on chernozem typical at Institute of Vegetables and Melons NAAS of Ukraine (Kharkiv oblast, Ukraine). Experimental field is located in Forest-Steppe zone of Ukraine (49°.45' N, 35°.51' E and 110 m above mean sea level). The territory of experimental fields is characterized by a temperate continental climate. The sum of positive temperatures is about 2850°C. The vegetation period (days with an average daily temperature above 5°C) is 195-220 days. The average annual precipitation is 560 mm.

Soil - chernozem typical heavy loamy with pH = 5.7, bulk density = 1.30 g cm^{-3}, the amount of absorbed bases is 26.0 meq per 100 g of soil, hydrolytic acidity - 2.8 meq per 100 g of soil, humus content = 4.3 %. At the beginning of field experiment soil contained 15.2 mg kg^{-1} available nitrogen (NH_4-N + NO_3-N), 106-119 mg kg^{-1} available phosphorus (P_2O_5) and 173 mg kg^{-1} potassium (K_2O).

Experimental plot were irrigated by sprinkler irrigation system during 47 years (2-4 times per year with the norm of 350-500 m^3 ha^{-1}). An each experimental plot was 58.3 m^2 with 4 replicates. In all variant of experiment plow tillage was applied.

The chernozem samples were taken from the depth of 0-25 cm. Sampling locations varied in terms of fertilization: 1) Without fertilizer (control); 2) Mineral fertilization system ($N_{60}P_{57}K_{50}$); 3) Organic fertilization system (manure 21 t ha^{-1}); 4) Organo-mineral fertilization system (14 t ha^{-1} of manure + $N_{60}P_{57}K_{50}$).

Crop rotation: barley - cucumber - winter wheat - onion - tomato - cabbage - beetroot. $N_{540}P_{510}K_{450}$ (mineral fertilization system), manure 189 t ha^{-1} (organic fertilization system), 126 t ha^{-1} of manure + $N_{330}P_{330}K_{450}$ (organo-mineral fertilization system) were applied for rotation.

Organic carbon content was determined by Tyurin method based on dichromate oxidation. Organic carbon content was re-calculated into humus using the mean coefficient (1,724). Humus composition was determined by Tyurin method according to Ponomareva-Plotnikova procedure (Ponomareva & Plotnikova, 1980). Different organic matter fractions were isolated: humic acid (HA), fulvic acid (FA), and humin. For humus fractional composition, the solutions of different NaOH concentrations were used for extraction: 0.1 M NaOH (room temperature); 0.02 M NaOH (hot extraction) also 0.05 M H_2SO_4 (for decalcitation, room temperature) at a soil solution ratio at 1:20. The extracted humic substances were then separated into humic and fulvic acid fractions by acidifying the extract to pH 1.3-1.5 using 0.5 M H_2SO_4 at 68-70°C and humic acids were separated by filtering. Separated humic acids were re-dissolved in 0.1 M NaOH solution. Some humic and fulvic acid solutions of each fraction were evaporated and oxidized. Carbon content in the fractions of humic and a fulvic acid was determined by the dichromate oxidation procedure.

HA extracts were transferred to cuvettes. A solution of 0.02 M of NaOH was used as the blank. The absorbance of solutions at wavelengths of 465 and 665 nm was measured. The color indexes (E4: E6) were calculated as the ratio of E465: E665 nm. Spectral properties of solutions were measured using an UV-Vis spectrometer (SF-26).

All measurements were performed in triplicate. Statistical analysis of variance was performed using Statistica 10 software.

Initial soil test information collected in 1967 from 0-25 cm (Table 1).

Table 1. Fractional composition of humus in chernozem typical before the experiment

Corg, %	HA-1, %	HA-2, %	HA-3, %	FA-1,%	FA-2,%	FA-3,%	% to Corg			HA: FA
							HA	FA	Humin	
2.85	0.18	0.58	0.73	0.09	0.32	0.21	52.3	21.8	25.9	2.41

RESULTS AND DISCUSSIONS

Continuous irrigation (47 years) resulted in considerable changes of organic C in the topsoil (0-25 cm) in chernozem typical. Soil organic carbon content was by 10 % less than in initial soil before irrigation.

An improved water supply leads, on the one hand, to an increased yeild therefore to a higher input of carbon into the soil in the form of roots and plant material. On the other hand, consequently higher soil moisture results in an increased microbial decomposition of soil organic matter (Trost et al., 2013).

Analysis by Condron with coathors revealed that amounts of SOC were significantly greater between the dry land (125.5 mg ha^{-1}) and irrigation treatment (93.0 mg ha^{-1}).

Nitrogen fertilisation promotes plant growth but may lead to a change in the carbon: nitrogen ratio and hence to a higher decomposability of SOC.

In present study fertilization promoted to less carbon loss and in organo-mineral fertilization system to C accumulation (by 9 %) compered to plots without fertilization.

An increase in organic carbon stocks in response to organic fertilization by irrigation was not limited to the surface soil, but it continued down the soil profile to a depth of 160 cm (Bughio et al., 2016).

The fractional composition of humus of chernozem heavy loamy after long agricultural use (47 years) was determined that under the conditions of irrigation and application of mineral and organic fertilizers. A tendency to increase the content of mobile fulvic acids of fraction I from 3.5 to 5.1% of Corg (Table 2) was observed. The content HA-1 remained at 1.2% of Corg.

The results of long-term investigations show that during irrigation the humus state of chernozems of the Steppe zone is met with significant transformation in the direction of decreasing the total humus content and its fulvization (Shedrin et al., 2017).

The systematic application of mineral fertilizer separately and in combination with manure under the organo-mineral fertilization system increased the content of the most mobile groups of humic substances. The use of manure twice per rotation resulted in a reduction of the content of the active components in the soil organic matter by 11% of Corg compared to the control. This indicates that mobile organic matter is rapidly mineralized and is a source of nutrients for crops.

The content HA-2 bound with calcium under the effect of mineral and organo-mineral fertilization systems was not changed significantly. By the organic fertilizer system it was observed an accumulation HA bound with calcium of up to 51% of C org. With the increasing HA the content FA-2 decreases accordingly.

Table 2. Fractional composition of humus in chernozem typical under irrigation and fertilization

Treatment	Corg, %	HA-1, %	HA-2, %	HA-3, %	FA-1, %	FA-2, %	FA-3, %	% to Corg			HA:FA
								HA	FA	Humin	
Without fertilizer (control)	2.57	0.03	0.86	0.41	0.16	0.09	0.26	50.6	19.8	29.6	2.6
Mineral fertilization system	2.44	0.03	0.86	0.38	0.20	0.11	0.40	52.0	29.1	18.9	1.8
Organic fertilization system	2.41	0.03	1.23	0.41	0.17	0.07	0.26	69.3	20.7	10.0	3.3
Organo-mineral fertilization system	2.82	0.03	0.98	0.35	0.17	0.1	0.25	48.2	18.4	33.3	2.6
$LSD_{0.05}$[1]	0.41	0.01	0.11	0.10	0.03	0.03	0.08	-	-	-	-

[1]LSD 0.05 - Least Significant Difference at p = 0.05

It was found that under the conditions of application of only organic fertilizers the content HA-3 increased from 16% of Corg in the control variant to 17% of Corg by the organic fertilization system. The mineral fertilization system tends to increase the content FA bound with the mineral part of the soil.

With a slight variation in the relative content of mobile HA the influence of an agrogenic factor of different intensity on the indicator HA-1: FA-1 which reflects the direction of the first stage of the humification process was observed (Ovchinnikova, 2019). By the indicator of the ratio HA-1: FA-1 which according to the variants of the experiment was from 0.24 to 0.35 the low intensity of the process of humification at the stage of formation HA was observed.

The ratio of HA-1: FA-1 which characterized the polymerization of humus structures in the second stage of humification. Under organo-mineral fertilization system increased from 9.6 (on control) to 9.9 under the mineral fertilization system decreased to 7.8 which indicated a decrease in the intensity of formation of high-molecular compounds from low molecular ones. The application of single organic fertilizers increased the expansion of the HA-2: FA-2 ratio to 17.6 due to the supply of fresh organic matter which is a source for the synthesis of young HA.

Soil before the setting of the experiment in the arable layer of soil was characterized by a ratio of the amount of HA: FA at the level of 2.4 despite the anthropogenic influence (application of organo-mineral and organic fertilization systems in irrigated vegetable-fodder crop rotation) type of humus remained humate. With long-term use of mineral fertilizers. there was a narrowing of the HA: FA ratio to 1.8 in which the type of humus changed to fulvate-humate which was a sign of the negative orientation of the humification process.

Under conditions of long-term agricultural use of chernozem heavy loamy in irrigated vegetable-fodder crop rotation (9 crops) under different agrochemical loading signs of degradation transformation of humus with unequal degree of expression at different levels of its organization were revealed.

Although the humus content remained at a high level (at least 4 %) in the arable soil layer, the losses were recorded ranging from 1.2 to 15.7% of Corg of the baseline to the experiment (Figure 1). Such losses are estimated to be relatively low.

Differences of humus state of chernozem heavy loamy in the layer of 0-20 cm under different agrochemical load were diagnosed by the complex of characteristics. Changes in the variants of the experiment in the form of a trend were observed at the maximum values of the fertility parameters.

The degree of humification of organic matter was characterized as very high by the investigated variants. among which was notable the organic fertilization system. With the high degree of humification of organic matter. a very low content of "free" HA was observed.

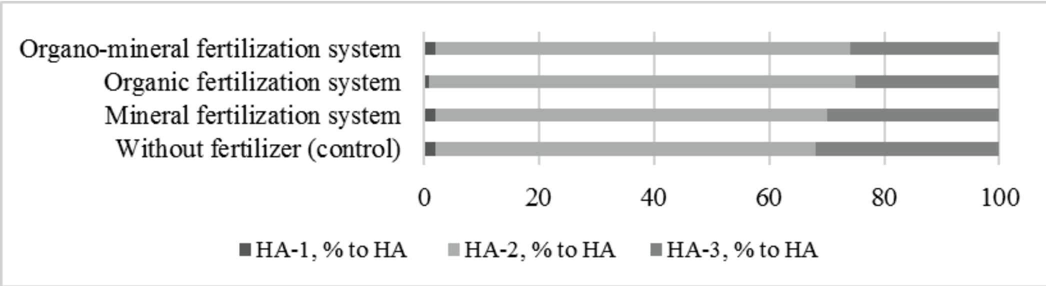

Figure 1. Content of different fractions of humic acids in chernozem typical under irrigation and fertilization

During the long agricultural use of chernozem heavy loamy the content of "free" HA in the arable layer of soil decreased by 5 times compared to 1967. The content HA bound with calcium from low level (soil before setting of the experiment) was changed to high according to the investigated variants among which were notable organic and organic-mineral fertilization systems. The content of strongly bound HA in the organic matter of chernozem heavy loamy remained high although changes in the direction of the conditions of humification were detected.

The primary information on the elements of humic substances was given by the values of E4: E6 ratio (ratio of the absorbances at 465 nm and at 665 nm. The E4: E6 ratio is considered to be inversely related to the degree of condensation and aromaticity of the humic substances and to their degree of humification (Senesi et al., 2003). The electronic absorption spectra of mobile fraction HA-1 at 465 nm and 650 nm are preferably lower than those of the other humus fraction Ca-bound fraction of humic acids (HA-2) and the fraction strongly bound with soil clay minerals (HA-3). Long-term agricultural use of chernozem heavy loamy without application of fertilizers resulted in a decrease in the E_4: E_6 ratio in labile humus compounds compared with soil before the experiment setting indicating that the aromatic nucleus with a condensed carbon atom network is predominant in the structure of HA-1 molecules.

The obtained data show that the organic matter of chernozem heavy loamy by different agrochemical loads has different degree of humification (Figure 2).

The decrease in molecular weight and carbon content the increase in the number of acidic functional groups and the oxidation of humic acids are observed under mineral and organo-mineral fertilization systems as evidenced by an increase of E_4: E_6 ratio by 38 and 67% compared to the control.

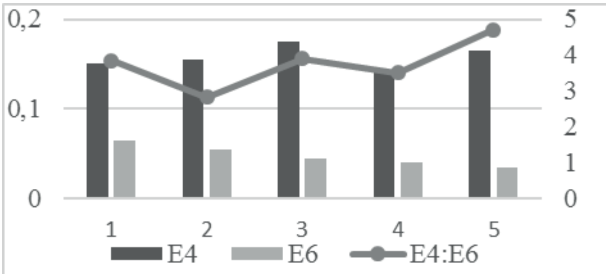

Figure 2. The ratio of the absorbances at 465 nm and at 665 nm of humic acids in chernozem typical under irrigation and fertilization

1 - Initial soil (before the experiment); 2 - Without fertilizer (control); 3 - Mineral fertilization system; 4 - Organic fertilization system; 5 - Organo-mineral fertilization system

The E4: E6 ratio was higher in organo-mineral fertilization system compared with the control and initial soil test mainly due to the higher proportion of fresh SOM. Mineral fertilization increases the content of aliphatic compounds in the humic substances while in organic fertilization system the E4: E6 ratio is lower which means a predomination of molecules with a high degree of aromaticy and condensation.

CONCLUSIONS

During 47 years of irrigation (≈ 2000 m^3 ha^{-1} $year^{-1}$) without fertilization a content of organic C in 0-25 cm of chernozem typical heavy loamy in a vegetable-fodder crop rotation have been decreased by 9.8% compared to initial data.

There were no statistically significant differences in soil organic C from mineral and organic fertilization systems and control (without fertilization).

Only organo-mineral system (126 t ha^{-1} of manure + $N_{330}P_{330}K_{450}$ per 9-course rotation) contributed to stabilization organic C content in chernozem typical under irrigation.

The topsoil highest humic acids (HA) content was observed under organic fertilization. Application of mineral fertilizers only led to increasing of the content of fulvic acids (FA) by 45% compared to other fertilization systems. Fractional composition of humus showed that 47 years of irrigation led to decrease of content of mobile humic acids (HA-1) in 6 times due to leaching while fraction of HA-2 increased by 50-112% compared to soil before irrigation that could be explain by intensive bounding with calcium from irrigation waters.

The intensity of the process of formation of humic acids and the process of polymerization of humus structures was evaluated according to the ratios HA-1: FA-1 and HA-2: FA-2 respectively. It was found that in organic fertilization humus structure is more compensated that in other fertilization systems.

On comparing E4: E6 ratios among different fertilization systems the lower ratio was in organic fertilization system which correspond with higher degree of aromaticity of organic substances in soil. Mineral fertilization increased the content of aliphatic compounds in the humic substances.

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CONSUMERS' PREFERENCES FOR PLACES TO PURCHASE LOCAL DAIRY PRODUCTS

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Abstract

Production and distribution of local food contributes to responds to the needs of consumers in terms of desire to support the local economies and responsibility for the environment. In local food resources, food production and processing, trading this food and its consumption occur in a relatively small geographical area. Benefits resulting from the functioning of local food systems are not only related to satisfying food demand, but also bringing specific social benefits. They allow to maintain the vitality of rural areas, especially in peripheral locations. In addition, locally produced food is not anonymous, it is also characterised by higher quality than mass-produced food. The purpose of the work is to identify the determinants and preferences of consumers regarding the places of purchase of local dairy products. The study was carried-out in 2019 using an interview questionnaire on a randomly selected group of respondents. The survey involved 308 respondents from the Podkarpackie Voivodeship, of which 298 consumed dairy products, and 255 respondents bought local dairy products (85.6% of respondents consuming dairy products). It was discovered the interest in buying local dairy products is quite high - 85.6% of respondents consuming dairy products bought them. A significant percentage of respondents (91.4%) indicated the availability of local dairy products in retail trade. Whereby, in the case of units located near the place of residence, 7.5% of respondents faced significant restrictions on access to these products, while 33.7% pointed to frequent shortages in the product range. This indicates the need to improve the flow of these products in distribution channels.

Key words: *local food, short supply chains, dairy products, consumers' preferences.*

INTRODUCTION

The food consumption sphere often becomes an opportunity to demonstrate specific views, the value system or opposition to the mass consumption model. Consumers make more and more conscious choices based on responsibility and benefits for the local community. For this reason, interest in local products and short food distribution chain in growing. Food from local production allows to implement the concept of sustainable rural development and sustainable consumption, contributes to reducing the negative effects associated with distribution and supports the development of local communities (Kusz, 2014). The development of local food distribution may become an important element of promoting the region, contribute to job creation and thus realistically support the development of the local economy (Cărătuș, 2018; Gómez et al., 2019; Gradziuk, 2015; Kacz, 2019; Kacz et al., 2018; Mikelionytė et al., 2019; Żakowska-Biemans et al., 2017).

In the source literature, the most commonly used criteria to qualify food in this category are the geographical place of manufacture and the perception of items by consumers as locally produced. "Local food systems" means that food production and processing, trade and consumption of food occur in a relatively small geographical area. Consumers most often interpret "local food" in reference to the distance that the food travels on the way from the producer to the consumer or the administrative boundaries within which it is produced (e.g. county, voivodeship, province). It should also be emphasized that "local food" is associated among consumers with certain attributes, as a product with unique features such as: freshness, high quality, animal welfare, environmental value, health values, compliance with sustainable production conditions, relationship with the local socio-cultural environment, which is the beneficiary of the development of such a concept of production and distribution (Kawecka and Gębarowski, 2015; Matysik-Pejas et al., 2017; Żakowska-Biemans et al., 2017)

Distribution channels are crucial in the development of the local food market. The institutional and functional structures of distribution channels may take various forms depending on the type of market. The choice of distribution channel is largely dependent on the type of product (and the degree of their processing) and the preferences of buyers (Nestorowicz et al., 2012). There may be various food distribution channels on the market of local food products, both traditional and modern (e.g. using the Internet, purchasing groups of consumers). Whereby, the availability of food products on the market largely depends on well-functioning distribution channels. In the process of distribution of local products, an important element is to determine consumer preferences regarding the choice of places to buy local food.

MATERIALS AND METHODS

The purpose of the work is to identify the determinants and preferences of consumers regarding the places of purchase of local dairy products.

The study was carried-out in 2019 using an interview questionnaire on a randomly selected group of respondents.

The survey involved 308 respondents from the Podkarpackie Voivodeship, of which 298 consumed dairy products, and 255 respondents bought local dairy products (85.6% of respondents consuming dairy products).

Detailed analysis were presented for respondents that declared to buy local dairy products. Articles produced in the Podkarpackie Province (the province located in the south-eastern region of Poland) were assumed as local dairy products.

The respondents were divided according to gender, age, education and place of residence. The structure of respondents is presented in Table 1.

Table 1. Characteristics of the respondents (%)

Characteristics	% of respond.	Characteristics	% of respond.
Gender	100.0	Education	100.0
-women	53.3	-primary	3.1
-men	46.7	-vocational	10.2
Age	100.0	-secondary	29.4
-up to 35 years	29.8	-higher	57.3
-36-45 years	23.5	Place of residence	100.0
-46-55 years	26.7	-city	28.2
-56 and more	20.0	-country	71.8

Source: own research

RESULTS AND DISCUSSIONS

Access to local food products on the market depends on well-functioning distribution channels.

Distribution is one of the most important elements of a company's marketing strategy. It is a set of activities that aim at moving products from the place of manufacture to the final consumer.

The main goal of distribution is to maximize the buyer's benefits, consisting in delivering the expected range of goods to the right place at the right time, with acceptable price and quality (Nestorowicz et al., 2012).

The availability of red goods sought by customers is both one of the basic dimensions of marketing effectiveness of enterprises, and also the most important element determining the purchase of food products.

Own research shows that only 8.6% of respondents had problems with buying local products in retail stores (Figure 1). Men, city dwellers, people aged 36-45 and respondents with primary education more often pointed out problems with purchasing local dairy products.

Further analysis pointed out the availability of local dairy products in retail units located close to the respondents' place of residence (Figure 2). A small percentage of respondents (7.5%) indicated that there was a lack of these products in retail stores in the place of their residence. The highest number of such respondents was among men, in the age group over 56 and among persons with vocational education.

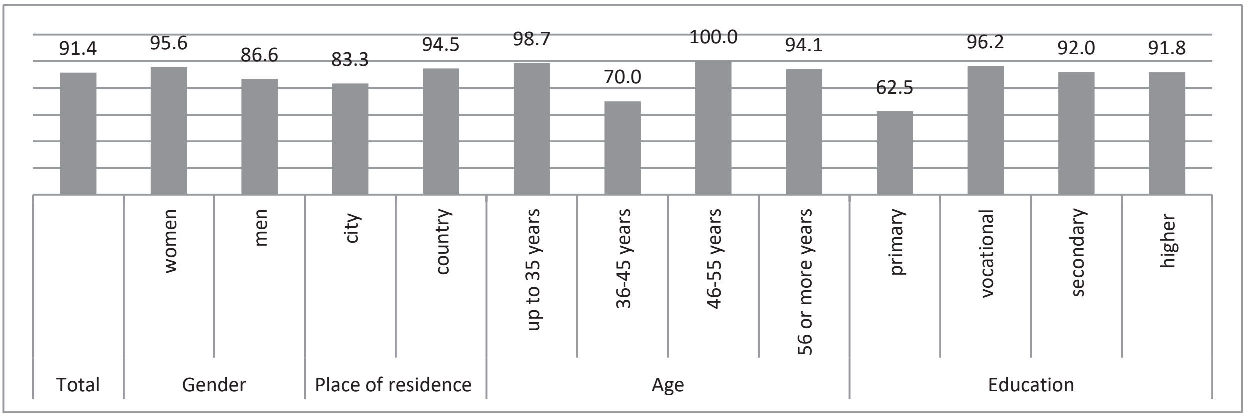

Figure 1. Percentage of respondents indicating availability of local dairy products in retail chains (%)
Source: own research

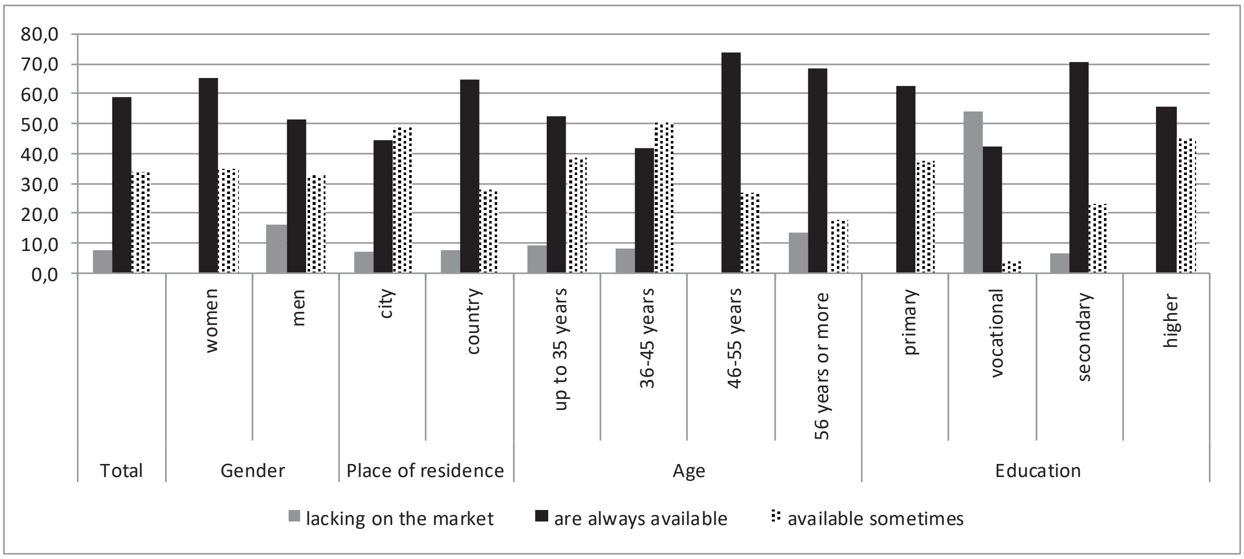

Figure 2. Degree of availability of local dairy products in the retail chains located in the place of residence (%)
Source: own research

Table 2. Place of purchase of local dairy products according to respondents* depending on gender and place of residence

Specification	**In total** *Frequency %*	**Gender**			**Place of residence**		
		Women	**Men**	***Chi-square test p-value***	**City**	**Country**	***Chi-square test p-value***
		Frequency %			*Frequency %*		
Small and medium shops	58.4	64.7	51.3	0.0297	52.8	60.6	0.2506
Super/hypermarkets, discount stores	16.5	17.6	15.1	0.5882	16.7	16.4	0.9578
Directly on a farm	14.1	-	30.3	0.0000	6.9	16.9	0.0391
Bazaars and marketplaces	15.7	17.6	13.4	0.3574	31.9	9.3	0.0000

*respondents could indicate more than one answer
Source: own research

Decisions regarding the places of purchase of food products taken by consumers are the result of many factors, i.e. the level of financial income, place of residence, age, education, their mobility, number of people in the household, distance from the sales point, convenience of shopping, availability of sought goods, attractive prices, convenient opening hours (Białobrzycka, 2012; Cyrek, 2012; Kusz et al., 2017; Łapińska, 2012). Analysing the preferences of places to buy local dairy products, respondents indicated that they most often purchase them in small and medium-sized grocery stores (Table 2).

Table 3. Place of purchase of local dairy products according to respondents* depending on age

Specification	Age				
	Up to 35 years	36-45 years	46-55 years	56 years and more	*Chi-square test p-value*
	Frequency %				
Small and medium shops	44.7	31.7	89.7	68.6	0.0000
Super/hypermarkets, discount stores	23.7	40.0	-	-	0.0000
Directly on a farm	31.6	8.3	-	13.7	0.0000
Bazaars and marketplaces	7.9	20.0	10.3	29.4	0.0044

*respondents could indicate more than one answer
Source: own research

Table 4. Place of purchase of local dairy products according to respondents* depending on education

Specification	Education				
	primary	vocational	secondary	higher	*Chi-square test p-value*
	Frequency %				
Small and medium shops	62.5	42.3	78.7	50.7	0.0003
Super / hypermarkets. discount stores	-	-	-	28.8	0.0000
Directly on a farm	-	53.8	21.3	4.1	0.0000
Bazaars and marketplaces	37.5	26.9	-	20.5	0.0001

*respondents could indicate more than one answer
Source: own research

In all distinguished groups of respondents, small and medium-sized stores were indicated as the most common place to buy local dairy products. The exception is the group of respondents aged 36-45, where shopping locations were more often indicated on super- and hypermarkets (Table 3). Analysing the impact of variables differentiating the respondents on decisions regarding the place of buying local food, it was found out that the gender of the respondents had a statistically significant influence of the choice of small and medium-sized stores, while it did not have a statistically significant impact on the choice of super and hypermarkets as the place to buy these products (Table 2). When analysing the impact of the place of residence on the selection of super- and hypermarkets as well as small and medium-sized stores, no statistically significant relationships were found (Table 2). In the case of age (Table 3) and education (Table 4), these relationships were statistically significant. In the aspect of local food products, an important issue is the possibility of offering them in short distribution chains. The role of transparency in the structure of such a food distribution channel is emphasized, which facilitates the identification of all participants in this chain, especially producers. The regional reach of short supply chains, promoting tourist attractions of the region, as well as the possibility of providing information about the nature and origin of products is also significant. The basic and easiest type of local food distribution channel is direct selling. It involves direct contact between the final consumer and the food producer, which can take place on the farm or marketplace, on the local food fairs organized cyclically or occasionally, as well as on exchanges or open-air market (Kawecka and Gębarowski, 2015). Alternative local food distribution channels, such as e-commerce, can also be mentioned.

When analysing the research results, it can be seen that the popularity of direct sales on a farm and purchases at bazaars and marketplaces was not high (Table 2). Most often men (Table 2), people up to 35 years of age (Table 3) and people with vocational education (Table 4) used direct sales on a farm. Statistical analysis confirmed the crucial statistical impact of gender, place of residence, age and education on the preferences for buying local products directly on farms. On the other hand, purchases of local dairy products at bazaars and marketplaces were popular among respondents living in the city (Table 2), aged 56 years and over (Table 3), and among people with primary and vocational education (Table 4).

Table 5. Local dairy products most often purchased in the opinion of respondents* differentiated in terms of gender and place of residence

Specification	In total	Gender			Place of residence		
		Women	Men	*Chi-square test p-value*	City	Country	*Chi-square test p-value*
		Frequency %			*Frequency %*		
milk	85.9	82.3	89.9	0.0836	83.3	86.9	0.4634
kefir	47.1	70.6	20.2	0.0000	61.1	41.5	0.0048
buttermilk	27.5	36.0	17.7	0.0010	18.1	31.1	0.0350
processed cheese	40.4	43.4	37.0	0.2982	34.7	42.6	0.2471
sour milk	22.3	32.4	10.9	0.0000	26.4	20.8	0.3319
butter	88.6	95.6	80.7	0.0002	83.3	90.7	0.0949
white cheeses	81.6	87.5	74.8	0.0090	84.7	80.3	0.4153
blue cheeses	21.6	26.5	16.0	0.0419	18.1	22.9	0.3923
yogurt	72.9	70.6	75.6	0.3659	76.4	71.6	0.4370
sour cream	69.4	78.7	58.8	0.0006	59.7	73.2	0.0352
yellow cheeses	67.1	69.1	64.7	0.4546	33.3	80.3	0.0000
Herbal cheeses	20.0	27.9	10.9	0.0070	27.8	16.9	0.0515

*respondents could indicate more than one answer
Source: own research

Table 6. Local dairy products most often purchased in the opinion of respondents* differentiated in terms of age

Specification	Age				
	Up to 35 years	36-45 years	46-55 years	56 or more years	*Chi-square test p-value*
	Frequency %				
milk	92.1	70.0	91.2	88.2	0.0008
kefir	28.9	30.0	75.0	56.9	0.0000
buttermilk	1.3	10.0	54.4	51.0	0.0000
processed cheese	40.8	21.7	36.8	66.7	0.0000
sour milk	7.9	20.0	47.1	13.7	0.0000
butter	92.1	80.0	92.6	88.2	0.0920
white cheeses	77.6	70.0	92.6	86.3	0.0061
blue cheeses	15.8	10.0	36.8	23.5	0.0013
yogurt	63.2	90.0	82.4	54.9	0.0000
sour cream	69.7	40.0	0.0	62.7	0.0000
yellow cheeses	55.3	60.0	89.7	62.7	0.0001
herbal cheeses	0.0	0.0	48.5	35.3	0.0000

*respondents could indicate more than one answer
Source: own research

Table 7. Local dairy products most often purchased in the opinion of respondents* differentiated diversified in terms of education

Specification	Education				
	primary	vocational	secondary	higher	*Chi-square test p-value*
	Frequency %				
milk	100.0	100.0	92.0	79.5	0.0049
kefir	62.5	0.0	46.7	54.8	0.0000
buttermilk	100.0	46.1	33.3	17.1	0.0000
processed cheese	37.5	57.7	38.7	38.4	0.3075
sour milk	0.0	26.9	33.3	17.1	0.0175
butter	100.0	80.8	100.0	83.6	0.0012
white cheeses	100.0	53.8	92.0	80.1	0.0001
blue cheeses	0.0	0.0	24.0	25.3	0.0126
yogurt	37.5	100.0	77.3	67.8	0.0006
sour cream	100.0	50.0	86.7	62.3	0.0001
yellow cheeses	100.0	50.0	76.0	63.7	0.0128
herbal cheeses	0.0	0.0	25.3	21.9	0.0172

*respondents could indicate more than one answer
Source: own research

A statistically significant impact of variables differentiating the respondents on purchasing decisions of local dairy products was found in bazaars and marketplaces, except for gender (Table 1).

Further analysis pointed out the local dairy products most frequently purchased by respondents (Tables 5-7). The highest percentage of respondents chose the following local dairy products: milk, butter, white cheese, yogurt, cream and yellow cheese (Table 5). The noticeable differences can be seen in consumer preferences regarding the choice of dairy products. Women much more often purchased local kefir, buttermilk, curd, butter, white cheese, blue cheese, cream, cheese with herbs than men, and these differences were statistically significant (Table 5). Also, the place of residence diversifies the choices of consumers. City dwellers were definitely more likely to buy local kefirs than country residents, while country dwellers were far more likely than city dwellers to reach for local products such as buttermilk, cream and cheese (Table 5). For other products, no statistically significant differences were noted. Analysing the responses that considered the age of respondents, it can be seen that in all age groups milk and butter purchased in local dairies were the most popular (Table 6). People aged 46 and older were more likely to buy kefir, buttermilk, blue cheese and herbal cheeses than younger people. A statistically significant impact of age on the selection of local dairy products was noted for all products except butter (Table 6). In the case of respondents diversified in terms of education, buttermilk was more likely bought by people with primary and vocational education, while this group of respondents did not buy cheeses with herbs (Table 7). In the case of other products, the attention should be drawn to the fact that people with vocational education did declare to purchase neither kefir nor blue cheese. The education of the respondents had a statistically significant impact on the selection of all analysed local dairy products except processed cheese (Table 7).

CONCLUSIONS

In the light of the above research, it should be stated that the interest in buying local dairy products is quite high - 85.6% of respondents consuming dairy products bought them. A significant percentage of respondents (91.4%) indicated the availability of local dairy products in retail trade. Whereby, in the case of units located near the place of residence, 7.5% of respondents faced significant restrictions on access to these products, while 33.7% pointed to frequent shortages in the product range. This indicates the need to improve the flow of these products in distribution channels. Most often, respondents pointed out small and medium grocery shops as a place where they buy local dairy products. Short distribution channels were not popular among the respondents. Rarely, the respondents used direct sales on a farm and purchases at bazaars and marketplaces. This may result from a small knowledge of these forms of sales and consumer concerns about the health safety of such food products. This requires educating consumers and informing about the benefits of shortening the food distribution chain.

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REMOVAL OF BASIC NUTRIENTS (NPK) BY APPLE TREES USING VARIOUS TYPES OF POTASSIUM FERTILIZERS IN THE CONDITIONS OF AZERBAIJAN

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Abstract

In the experiment field on irrigated meadow - brown soil was found that at low soil provision of exchangeable potassium in apple orchards applying of different types of potash on a background of organic fertilizer (20 t ha^{-1} of manure), significantly increases the accumulation of the main nutrient elements in leaves and fruits, and also takeaway NPK with apple harvest (classical orchard - 'Golden Delicious' variety and intensive orchard - 'Fuzhi' variety). The best variant was the sharing of organic fertilizer system (20 t ha^{-1} of manure - as background) with the high dose of potassium sulphate (K_{160}): compared with the control variant (without fertilizers), the yield was higher by 9.9 t ha^{-1} or 7.87%, and the removal of nitrogen, phosphorus and potassium from the soil - by 69.0 kg ha^{-1}, 46.3 kg ha^{-1}and 120.4 kg ha^{-1}, respectively; and compared with the background + K_{160} potassium chloride variant, these indicators were higher by 0.95 t ha^{-1}, or 4.2%, respectively: 6.2 kg ha^{-1}, 7.1 kg ha^{-1} and 14.2 kg ha^{-1}. This variant was also effective in relation to the accumulation of nitrogen, phosphorus and potassium in the leaves and fruits of the apple trees, as well as the removal with harvest in classical orchard. Similar results were obtained using organo -mineral system fertilizer - 20 t ha^{-1} manure + K_{160} potassium sulphate also in intensive orchard.

***Key words**: forms of potash fertilizers, NPK removal, apple yield, irrigated meadow-forest soils, fertilizer system.*

INTRODUCTION

In orchards, the removal of nutrients is determined by the magnitude of the vegetative mass of trees. With the entry into the fruiting season and a significant decrease in tree growth strength, the takeaway is determined mainly by the fruit yield. Changes in the nutritional conditions have a significant effect on the absorption and alienation of nutrients from the soil. Knowledge of apple growing conditions, meteorological conditions of the year, type of soil, degree of fertilizer allows you to get a more complete picture of the quantitative changes in the alienation of nutrients from the fruit harvest (Akhmedov, 1989; Vitovskaya, 2015; Volodina et al., 2014).

Establishing the nature of the impact of growing conditions on the change in the ratio and amount of nutrients alienated from the soil, including nitrogen, phosphorus and potassium, provides information on the alienation of nutrients. This makes it possible to obtain a more complete picture of the nutrient requirements of trees, which is important for solving the problems of building fertilizer systems in tree orchards. The removal of chemical elements from the soil by plants depends on the intensity of their entry into plants and the growth rate of biomass. It is known that this indicator varies widely depending on the species, varietal characteristics and growing conditions of the crops. The accumulation of chemical elements by plants, in addition to species and varietal characteristics of plants, depends on the phase of their development, on the soils buffering ability, the properties of chemical elements and the forms of their presence in the soil (Krivoruchko, 1978; Kushnirenko, 1978; Kushnirenko & Razumnaya, 1978; Rubin & Moiseenko, 1970).

The growth rate of plant biomass depends significantly on the content of nutrients in the soil. Fertilizers application affects the productivity of crops, increasing both biomass and the removal of chemical elements by the crop (Rubin & Moiseenko, 1970; Semchenko & Sinitsin, 1977; Vitovskaya, 2015).

A significant number of studies carried out in various regions are devoted to studying the influence of various fertilizers systems, as well as species, doses and forms of mineral fertilizers on the productivity and quality of apple trees (Akhmedov, 1989; Lapa et al., 2009; Volodina et al., 2014; Yakimenko, 2017). The difference in the results obtained is probably due to differences in the soil-climatic and agrotechnical conditions of the experiments.

The aim of the reserches is to study increasing doses of various forms of potash fertilizers (against the background of organic fertilizers 20 t ha^{-1} of manure) on the yield of the apple tree and on the accumulation of essential nutrients (NPK) in the fruits and leaves of the apple tree, their removal from the plant (crop) under conditions of the irrigated meadow-brown soils of Azerbaijan. The relevance of these works has recently been increasing due to the low and unbalanced use of increasing doses of mineral fertilizers in the region's agriculture (Akhmedov, 1989; Mammadov, 1980; Mammadov et al., 2012).

MATERIALS AND METHODS

The studies were carried out on the irrigated meadow-brown soil of the dry subtropics of the north-eastern part of the Greater Caucasus within Azerbaijan, in the experimental base of the Research Institute for Fruit Growing and Tea Production of the Ministry of Agriculture of Azerbaijan, village Zardobi, Guba district.

Climate of Guba - Khachmaz zone differs sufficiently of aridity: the average annual precipitation - 350 mm, the average temperature - 13.8^{0}C, the amount of active air temperature > 10^{0}C - 4000. In the experimental variants were used the following types and fertilizers system: mineral - $N_{aa,}P_{sp},K_x,K_s$; organic - cattle manure semi-rotted, 65% of humidity (Dospehov, 1985; Guidance..., 2008).

The test was performed in the classical orchard - old planting for 30 years, apple variety 'Golden Delicious' (scheme 8 m x 4 m) and intensive orchard - planting recent 4-year-old, apple variety 'Fuzhi' (scheme 4 m x 1 m). The total area of the classical orchards - 9600 m^2, intensive orchards - 1200 m^2. The experiments were performed in triplicate variants.

Soil characteristics of the experimental plot (irrigated meadow-brown soils) before laying the experiment: soil reaction - slightly alkaline (pH-7.8), low content (16.8 mg kg^{-1}) of mobile forms of P_2O_5 (according to Machigin) and an average content of exchange forms K_2O - 238 mg kg^{-1} of soil. The amount of humus in the arable layer is 3.25%. The amount of ammonia nitrogen ($N\text{-}NH_4$) - 17.5; nitrate nitrogen ($N\text{-}NO_3$) - 9.62 mg kg^{-1} of soil (Agrochemical..., 1975; Arinushkina, 1970; Dospehov, 1985).

The following fertilizers were used: nitrogen fertilizers - ammonium nitrate NH_4NO_3 (N-34%); phosphate - simple superphosphate - $Ca(H_2PO_4)_2$ (P_2O_5- 18%), and from potassium - potash chloride KCl (K_2O-46%) and potash sulphate K_2SO_4 (K_2O-52%). Organic fertilizer - the semi-rotted manure cattle (humidity 65%), the average content of nitrogen - 0.5%, P_2O_5 - 0.25% and K_2O - 0.58%.

The entire annual rate of organic fertilizers (100%) was ploughed in the soils. Mineral fertilizers were applied in three periods during the growing season of the apple trees in the following phases of plant development: during shoot swelling; after flowering; when setting fruits (Dospehov, 1985; Vitovskaya, 2015).

In field studies, according to the experimental design, the following mineral, organic, and organo – mineral fertilizers systems were used:

1. Control variant (without fertilizers);
2. Cattle manure 20 t ha^{-1} - Background or Fond - organic fertilizers system;
3. Background + K_{140} (KCl) - organo - mineral fertilizer system;
4. Background + K_{140} (K_2SO_4) - organo - mineral fertilizers system;
5. Background + K_{160} (KCl) - organo - mineral fertilizers system;
6. Background + K_{160} (K_2SO_4) - organo - mineral fertilizers system;
7. $N_{100}P_{50}K_{120}$ - mineral fertilizers system.

Before laying the experiment, an agrochemical examination of the arable soil layer of the experimental plots was carried out. For this purpose, the following analyses was determined: the humus content according to Tyurin in the modification (GOST 26213-91), the nitrate nitrogen - ionometric method, the ammonium - colorimetric method with reagent

Nessler, mobile forms of the nutrients - in an extract Machigin: phosphorus - colorimetric method, potassium exchange - flames hydrochloric photometry method (GOST 27207-91). The pH of the water determined potentsiometrical method - GOST 26483-85 (Agrochemical..., 1975; Arinushkina, 1970). Accounting the crop yield and leaves was carried out by the gravimetric method - fresh and air-dry (Derzhavin, 1999; Dospehov, 1985; Guidance..., 2008).

In plant samples (leaves and fruits), the content of total forms of nitrogen, phosphorus and potassium was determined by Ginzburg G.E., Scheglova G.M. and Wulfius E.V. (Arinushkina, 1970). Plant samples were taken from each plot from all variants at the time of selection of soil samples (Derzhavin, 1999).

Effect of different doses of mineral and organic fertilizer, as well as their combined application to the takeaway of macro elements by apple tree, was studied in 2017-2019 years in the conditions of stationary field experience.

RESULTS AND DISCUSSIONS

Currently, one of the possible reserves for increasing soil fertility and cultivation is organic fertilizers. Numerous researchers have provided the high efficiency of manure and its positive role in the increasing of soil fertility, however, comprehensive studies on the effect of various types of organic fertilizers in comparison with mineral fertilizers are not enough (Ermakova & Novikov, 2019; Merzlaya, 2006; Volodina et al., 2014).

Identification (clarification) of factors affecting the ratio of nutrients alienated from the crop and contained in fertilizers is of great importance when choosing a fertilizer system in a crop rotation. The issue of doses and ratios of mineral and organic fertilizers, in connection with the thickened methods of growing apple trees and intensifying gardening, continues to be one of the central issues in the fertilizer system of orchards (Kushnirenko, 1978; Kushnirenko & Razumnaya, 1978; Semchenko & Sinitsin, 1977). One of the mandatory factors for the effective and sustainable functioning of agrocenoses is the rational use of mineral fertilizers, which allows to obtain at least 30-50% of additional yield increase with good quality while preserving and increasing soil fertility (Volodina et al., 2014).

In addition, the preservation and increase of the fertility of irrigated meadow-brown soils is directly related to the use of organic, organo-mineral and mineral fertilizers, which have a significant impact on its agrochemical properties. In most cases, their effect on the soil properties is positive and contributes not only to its stabilization, but also for improvement the fertility parameters (Ermakova & Novikov, 2019; Nikitishen & Demidov, 1987).

The research scheme provided for the establishment of a possible depletion or enrichment of this soil with basic nutrients, the study of the comparative effect of mineral and organic fertilizers, as well as their joint application, the use of mineral fertilizers in quantities equivalent to the content of basic nutrients in manure or half the nitrogen content of manure, as well as the effectiveness of the joint application of manure and mineral fertilizers in apple orchards, supplementing manure with individual nutrient exists (Guidance..., 2008; Mammadov et al., 2012; Merzlaya, 2006; Semchenko & Sinitsin, 1977).

An analysis of data conducted by various authors of the studies shows that the use of different types of potash fertilizers in the mineral, organic, and organic-mineral fertilizer systems play a significant role in the accumulation of nitrogen, phosphorus, and potassium in individual organs of the apple tree plant (Krivoruchko, 1978; Shafran et al., 2012; Yakimenko, 2017).

The effect of two types of potash fertilizers on the accumulation of the main nutrient elements (NPK) of apple trees under irrigated meadow-brown soils was studied. The average data for 3 years is shown in Figure 1.

The figure shows that the use of various types of potash fertilizers in the mineral and organic systems had a different effect on the accumulation of basic nutrients in the above ground organs (leaves and fruits) of apple trees. It was found that increasing the doses of various fertilizer systems and the intensity of using different types of potash fertilizers according to the experimental variables correspondingly affected the fertilizer efficiency in accumulating nutrients in the leaves and fruits of the apple tree.

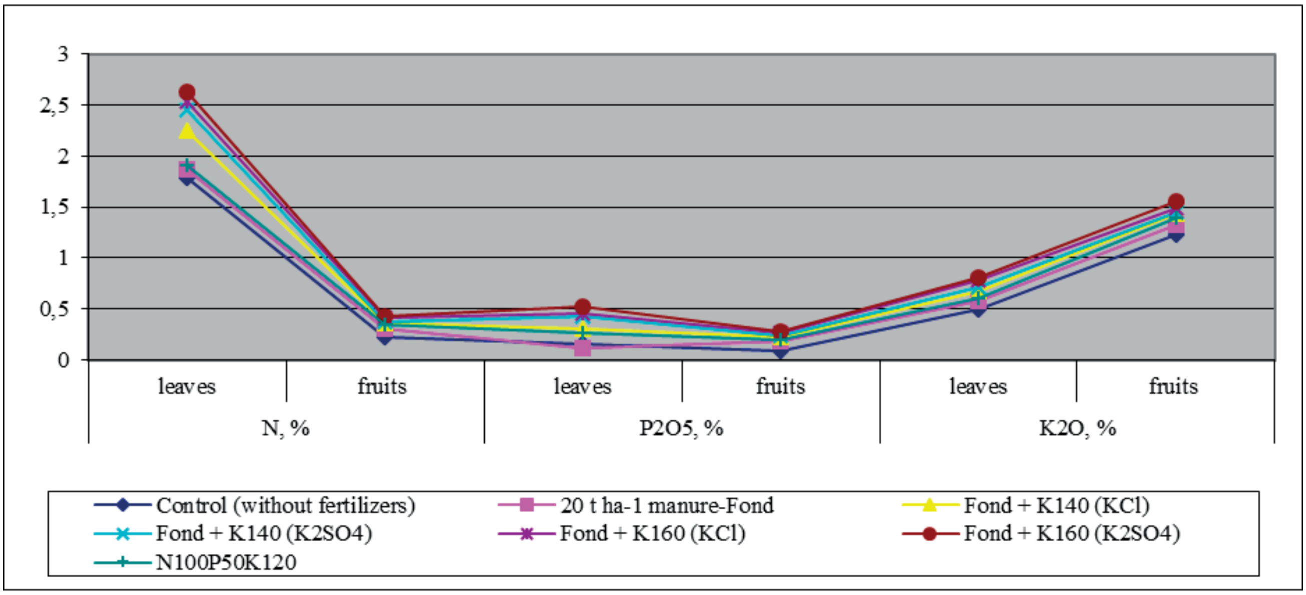

Figure 1. The influence of different forms of potash fertilizers on the accumulation of nutrient elements (NPK) in individual organs of the apple tree ('Golden Delicious')

Thus, in the control (without fertilizers) variant, the accumulation of total nitrogen in apple leaves is 1.79%, and in fruits 0.22%, and in the application of the organic fertilizer system (20 t ha^{-1} of manure). These data were 1.87 and 0.31%, respectively, and in the mineral fertilizer system ($N_{100}P_{50}K_{120}$) - 1.91 and 0.34%, while the organic fertilizer system combined the use of different types of potash fertilizers with organic fertilizers resulted in a much greater accumulation of total nitrogen in apple organs. It was greatest when potash fertilizers were used as potassium sulphate in the dose of 20 t ha^{-1} K_{160} manure variant, where these data reached 2.63 and 0.43% respectively, which exceeded the 20 t ha^{-1} K_{160} manure variant (potassium chloride) by 0.09% and 0.01%.

The accumulation of total nitrogen in apple leaves and fruits increased by 0.08% and 0.09%, respectively, compared to the control in the organic fertilizer system, and the total nitrogen increased by 0.12% in the leaves and fruits when an equivalent amount of mineral fertilizers was used in dose of $N_{100}P_{50}K_{120}$.

The use of potassium fertilizers in the form of potassium sulphate in the organo-mineral system, in dose of 20 t ha^{-1} of manure + K_{160} increased the amount of nitrogen, phosphorus and potassium in the leaves and fruits, respectively, by 0.084% and 0.21% nitrogen; 0.37% and 0.19% of phosphorus; and 0.32% and 0.32% of potassium.

The effectiveness of the use of potash fertilizers in conjunction with organic fertilizer (organic-mineral fertilizer system), mainly depended on the size of their doses, as well as on different types of potash fertilizers. At low (K_{140}) and high (K_{160}) doses of potash fertilizers against organic ones, the potassium sulphate efficiency slightly exceeded the potassium chloride efficiency. In the case of using increased doses of potassium fertilizers (K_{160}), potassium sulphate was clearly preferable for the accumulation of essential nutrients (NPK) in the leaves and fruits of the apple tree.

Perhaps this is due to the physiological role of sulphur in the plant. Sulphur is an important element of plant nutrition; its excess is more harmful to plants than a deficiency.

Our studies also revealed the influence of different types of potash fertilizers on the accumulation of nutrients in individual organs of the apple tree in young orchards with 'Fuzhi'variety (Table 1).

Similar results were also obtained during the experiment in intensive orchards (variety 'Fuzhi'). So, in the fruits of intensive orchards, using different types of potash fertilizers against organic (20 t ha^{-1} of manure), the most effective was the use of potassium sulphate in the dose of K_{160}.

The best experience was found to be the joint use of an organic fertilizer system (20 t ha^{-1} of manure - background) with an increased dose (K_{160}) of potassium sulphate: here, compared with the control variant without fertilizers, the yield was higher by 9.9 t ha^{-1} or 7.87%, and the removal of nitrogen, phosphorus and potassium from the soil - respectively, 69.02 kg ha^{-1}, 46.3 kg ha^{-1} and 120.41 kg ha^{-1}; and compared with the Background + K_{160} potassium chloride variant, these indicators were higher by 0.95 t ha^{-1} or 4.2%, respectively; 6.2 kg ha^{-1}, 7.06 kg ha^{-1} and 14.15 kg ha^{-1}.

Table 1. The influence of different forms of potash fertilizers on the accumulation of nutrient (NPK) elements in individual organs of the apple tree (cultivar 'Fuzhi')

No	Experimental Variants	The dry matter NPK content					
		N, %		P_2O_5, %		K_2O, %	
		Leaves	fruits	leaves	fruits	leaves	fruits
1	Control (without fertilizers)	1.85	0.26	0.13	0.17	1.28	0.53
2	20 t ha^{-1} cattle manure - background (fond)	1.94	0.33	0.18	0.22	1.37	0.64
3	Background + K_{140} (KCl)	2.48	0.38	0.39	0.24	1.48	0.70
4	Background + K_{140} (K_2SO_4)	2.59	0.41	0.51	0.25	1.52	0.73
5	Background + K_{160} (KCl)	2.67	0.44	0.59	0.29	1.58	0.82
6	Background + K_{160} (K_2SO_4)	2.75	0.46	0.63	0.30	1.61	0.85
7	$N_{100}P_{50}K_{120}$	2.16	0.36	0.56	0.23	1.42	0.67

This option was also effective against the accumulation of nitrogen, phosphorus and potassium in the leaves and fruits of the tree.

NPK carry-out rates by individual plant organs are a mirror reflection of the ratios in the fertilizer system. The study of total nutrient withdrawal in order to establish a link between soil fertility and crop production is a priority for agrochemical research. The removal of nutrients from the soil depends, as you know, on the level of their content in the main products, as well as productivity (Rubin & Moiseenko, 1970; Semchenko & Sinitsin, 1977; Vitovskaya, 2015; Zelenskaya, 1969).

With the use of different types of potash fertilizers in the mineral, organic, and organo - mineral systems, the removal has increased in all variants, that is, the alienation of the main nutrients from the soil with the crop by the apple tree plant 'Golden Delicious' variety (Figure 2).

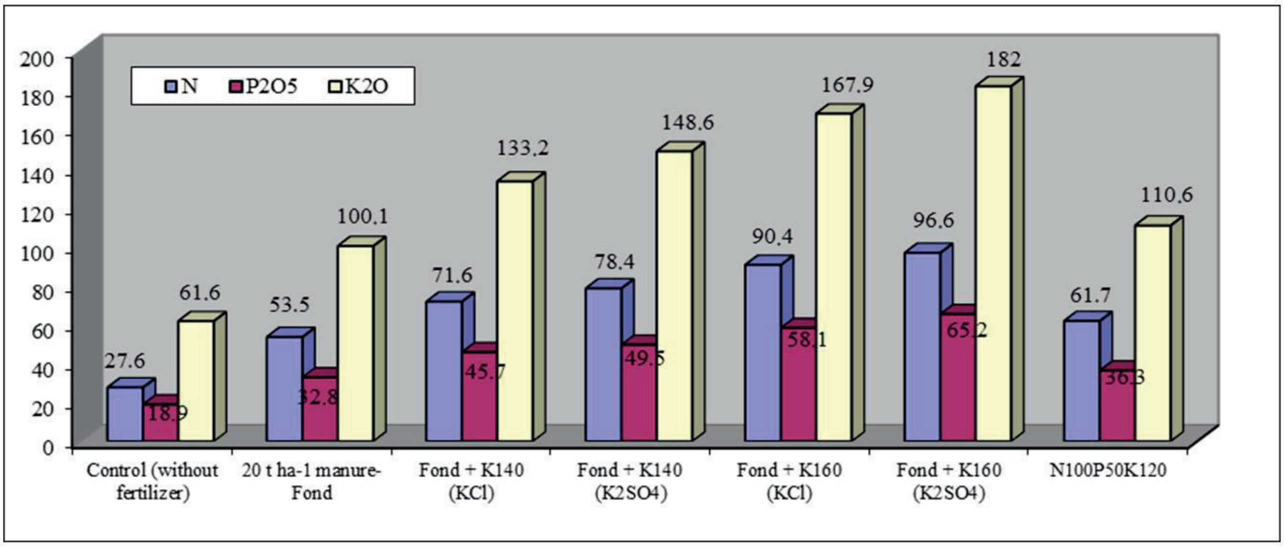

Figure 2. The influence of different forms of potash fertilizers on takeaway of NPK by apple fruits (variety 'Golden Delicious')

So, in the control, non-fertilized variants, the removal of nitrogen, phosphorus and potassium with the apple crop was, respectively: 27.6; 18.85; 61.59 kg ha^{-1}.

These indicators increased with the use of an organic fertilizer system in the background variant at a rate of 20 t ha^{-1} of manure and were 25.8 kg ha^{-1} more nitrogen compared to the control variant; 13.9 kg ha^{-1} for phosphorus and 38.4 kg ha^{-1} of potassium.

When applying the equivalent amount of mineral fertilizer to 20 t ha^{-1} of manure in the dose of $N_{100}P_{50}K_{120}$, these indicators were higher compared to the control variant by 34.07 kg ha^{-1} for nitrogen, 17.43 kg ha^{-1} for phosphorus and 49.06 kg ha^{-1} for potassium. The use of two types of potash fertilizers - potassium sulphate and potassium chloride against the background of organic fertilizers (organo-mineral fertilizer system) had a different effect on the removal of nutrients from the apple crop in classical orchards. At low (K_{140}) and increased (K_{160}) doses of potash fertilizers, potassium sulphate exceeded potassium chloride in the removal of nitrogen, phosphorus and potassium. So, in the variant Background (20 t ha^{-1} of manure) + K_{140}(KCl) the nitrogen removal with the apple crop is 71.56 kg ha^{-1}, phosphorus - 45.72 kg ha^{-1}, potassium - 133.19 kg ha^{-1}.

When replacing potassium chloride (KCl) with the same amount of potassium sulphate (K_2SO_4), the nutrient removal (NPK) significantly increased the nitrogen to 78.43 kg ha^{-1}; for phosphorus up to 49.53 kg ha^{-1} and for potassium up to 148.60 kg ha^{-1}, that is, amounted to 6.24 kg ha^{-1}, 7.0 kg ha^{-1} and 14.15 kg ha^{-1}, respectively.

At high doses (160) of various forms of potash fertilizers (KCl and K_2SO_4) against the background of organic (20 t ha^{-1} of manure) in the organic-mineral fertilizer system, these indicators significantly increased.

So, in the variant Background + K_{160} (K_2SO_4), the nutrient removal increased in comparison with the control variant - nitrogen by 62.02, phosphorus by 45.31, potassium by 120.41 kg ha^{-1}; and in comparison with the Background + K_{160} (KCl) option, 6.24, 7.06 and 14.15 kg ha^{-1}, respectively. Similar results were obtained from the experience carried out in an intensive orchards of 'Fuzhi'apple variety (Table 2).

Table 2. Influence of different forms of potash fertilizers on the yield and NPK removal by fruits of apple trees 'Fuzhi'

No.	Experience Variants	Productivity, t ha^{-1}	Increase		Removal, kg ha^{-1}		
			t ha^{-1}	%	N	P_2O_5	K_2O
1	Control (without fertilizers)	7.64	-	-	19.87	12.98	40.49
2	20 t ha^{-1} cattle manure - background (fond)	12.76	5.12	67.0	42.10	28.07	81.66
3	Background + K_{140} (KCl)	14.87	7.23	94.6	56.50	35.68	104.10
4	Background + K_{140} (K_2 SO_4)	15.24	7.60	99,4	62.48	38.1	111.25
5	Background + K_{160} (KCl)	16.57	8.93	116.8	72.90	46.39	135.87
6	Background + K_{160} (K_2SO_4)	17.15	9.51	124,4	78.89	51.45	145.77
7	$N_{100}P_{50}K_{120}$	13.23	5.59	73.16	47.62	30.43	88.64

Here, on the variant with the use of potassium sulphate against the background of organic fertilizers was also established as an effective effect: the removal of nitrogen, phosphorus, and potassium increased by 59.02 kg ha^{-1}, 38.47 kg ha^{-1} and 105.28 kg ha^{-1}, respectively; compared with the control and, respectively, 5.9 kg ha^{-1}, 5.06 kg ha^{-1} and 9.9 kg ha^{-1} compared to the Fond + K_{160} (KCl) option.

Thus, the effectiveness of the organo-mineral fertilizer system has been identified. It was found that the use of potassium sulphate at a rate of 160 kg ha^{-1} against a background of organic (20 t ha^{-1} of manure) fertilizers is more effective. The effect of different types of potash fertilizers on the 'Golden Delicious' apple yield in irrigated meadow-brown soil was also studied (Figure 3).

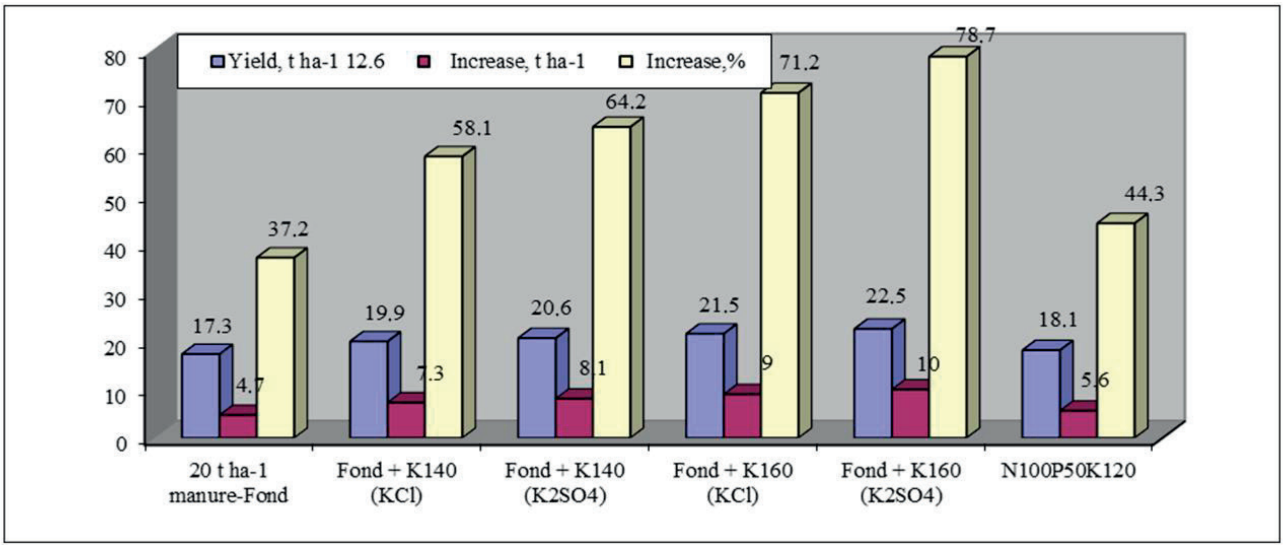

Figure 3. Influence of different forms of potash fertilizers on the yield and fruits of the apple tree 'Golden Delicious'

From the presented data it is seen that the use of various types of potash fertilizers on the background of organic fertilizers (20 t ha^{-1} of manure) affects the yield of apple trees in different ways. If in the non-fertilized (control) variant, the apple tree yield reaches up to 12.57 t ha^{-1}, then with the use of various fertilizer systems these indicators reach from 17.25 t ha^{-1} to 22.47 t ha^{-1} according to the experimental options. At the same time, the increase in yield by fertilized options varies from 4.68 t ha^{-1} or 37.2% to 9.90 t ha^{-1} or 78.7%.

When applying the organic systems fertilizer (20 t ha^{-1} manure), in the conducted experiment in classical orchards 'Golden Delicious' compared with the control, harvest of apple was 5.12 t ha^{-1} or 67.0%; when using a mineral fertilizer system equivalent to 20 t ha^{-1} of manure ($N_{100}P_{50}K_{120}$), the increase was 5.59 t ha^{-1} or 73.16%.

The use of different types of potash fertilizers in the norm of K_{140} and K_{160} against the background of the organic system (20 t ha^{-1} of manure) fertilizers also affects the yield of apple trees in different ways. At lower rates (K_{140}), the use of potassium chloride and potassium sulphate together with organic fertilizer (20 t ha^{-1} of manure) yield apple trees reached 19.88 and 20.64 t ha^{-1}, respectively. The increase was 7.31 and 8.07 t ha^{-1}, or 58.1% and 64.2% compared with the control (not fertilized) option. In these options, potassium sulphate is installed more efficiently from potash fertilizers, which exceeds potassium chloride by 0.37 t ha^{-1} or 2.4%.

And at elevated rates (K_{160}), the use of various types of potash fertilizers together with organic fertilizers (20 t ha^{-1} manure), that is, with the organo-mineral fertilizer system, the apple tree yield increased to 22.47 t ha^{-1}. This is the highest result of the field experience. At the same time, the increase in yield compared to the control was 9.90 t ha^{-1} or 78.7%, and the increase compared to the Fond + K_{160} (KCl) option was 0.95 t ha^{-1} or 4.2%. Similar results were obtained from the field study carried out in an intensive apple orchards 'Fuzhi' variety.

So, in experiments conducted in intensive orchards using different forms of potash fertilizers, a more effective option was established with the use of sulphate (K_{160}) against the background of organic fertilizer (20 t ha^{-1} manure). In this embodiment, the yield of apple increased by 0.58 t ha^{-1} or 3.4% compared with the Fond + K_{160} (KCl).

Thus, it has been found that the most effective use of potassium sulphate in conjunction with organic fertilizers, i.e. the organic-mineral fertilizer system.

CONCLUSIONS

As shown by the experiments, the percentage of the staple elements in different organs of apple has a specific value, characteristic of the body, it depends of the fertilizer.

The effect of fertilizers on increasing the percentage of nitrogen was noted significantly more in the leaves compared with the fruits of the apple tree. When applying various fertilizer systems, the increase in nitrogen and phosphorus compared to the non-fertilized version in the classic orchards 'Golden Delicious' varies between 0.3-0.4% and 0.08 - 0.84%, respectively, and in intensive orchards 'Fuzhi' - 0.09-0.9% and 0.05-0.5%.

The influence of various fertilizer systems of increasing the percentage of potassium was revealed in the leaves and fruits of the apple tree: in comparison with the control version, in the organic fertilizer system in classical orchards site was 0.09%, in intensive - 0.14%, and in the variant 20 t ha^{-1} of manure + K_{160}(K_2SO_4), respectively, 0.38 and 0.32%.

The results of the experiments showed that the removal of nutrients depends not only on the size of the crop, but also on their balance in the nutrient medium. The use of fertilizer systems, depending on the different types of potash fertilizers, affects the removal of the main nutrients with the apple crop in different ways. The largest takeaway in the experiment per unit area was noted when applying the organo-mineral fertilizer system (20 t ha^{-1} of manure + K_{160}) in classical orchards. Moreover, in the composition of the organic-mineral fertilizer system from mineral fertilizers, potassium sulphate was more effective than potassium chloride. In this embodiment, NPK was established with a yield of 96.62 kg ha^{-1} for nitrogen, 65.16 kg ha^{-1} for phosphorus and 182.00 kg ha^{-1} for potassium. At the same time, the increase in NPK removal was in this variant compared to the control by 69.02, 46.30 and 120.41 kg ha^{-1}, respectively. The removal in classical orchards was higher compared to the control variant for nitrogen, phosphorus and potassium, respectively, by 34.07 kg ha^{-1}, 17.43 kg ha^{-1} and 49.06 kg ha^{-1} with the mineral fertilizer system; 25.87 kg ha^{-1}, 13.92 kg ha^{-1} and 38.46 kg ha^{-1} with an organic fertilizer system (20 t ha^{-1} of manure). Harvesting in intensive orchards 'Fuji' had similar results, i.e. was higher when making K_{160} in the form of potassium sulphate against the background of organic fertilizers. Here, potassium sulphate was also more effective than potassium chloride. And compared with the use of the same doses of mineral and organic fertilizers (in 20 t ha^{-1} of manure + K_{160} (KCl), the nitrogen increased by 69.0 kg ha^{-1}, phosphorus by 46.3 kg ha^{-1} and potassium -120.4 kg ha^{-1}.

Separate, as well as joint application of mineral and organic fertilizers on irrigated meadow-brown soils significantly affects the productivity of apple trees. The best results were obtained in both experiments (orchards) with utilization of potassium fertilizers in high doses of K_{160} in the form of potassium sulphate against organic (organic-mineral fertilizer system). In the variant - 20 t ha^{-1} of manure + K_{160} (K_2SO_4), the apple yield ('Golden Delicious' variety) increased by 99.0 kg ha^{-1} or 78.7% compared with the control variant. The use of sulphuric acid potassium (K_{160}) together with 20 t ha^{-1} of manure was more effective than the use of potassium chloride (K_{160}). In these options, the difference in apple yield was 0.95 t ha or 4.2%. In intensive apple orchards, high yields on apple were also found in the 20 t ha^{-1} of manure + K_{160} (potassium sulphate). In this case, the increase amounted to 95.1 kg ha^{-1} or 124.4% compared with the control version, and compared to the same dose of potassium chloride against the background of 20 t ha^{-1} of manure by 5.8 t ha^{-1} or 3.38%.

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THE INFLUENCE OF EPIDEMICS ON TOURISM UNDER THE CONDITIONS OF GLOBALIZATION

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Abstract

Tourism is one of the most important sectors of the economy of a country, but any event with negative connotations such as the appearance of epidemics, terrorist attacks, economic or social instability influences the activity in this sector. In the context of the current crisis triggered by the onset of coronavirus (COVID - 19) and based on past experiences, in this paper we intend to analyse how in the last 20 years, different epidemics have contributed to reducing tourism in the affected areas and to economic losses important. The paper tests by empirical analyses based on the data published in different international databases, as well as by a quantitative analysis how the appearance of epidemics influences the tourism in both the affected areas, as well as worldwide.

***Key words**: tourism, epidemics, COVID-19, revenues, tourist circulation.*

INTRODUCTION

A study by the World Travel & Tourism Council shows that various events that take place in the world negatively influence the tourism market, due to the consequences they have on the collective memory. If the return to the initial values takes place after 13 months in the case of terrorist attacks, in the case of an epidemic the decrease of the tourist circulation is maintained for a period of 21 months. Therefore, the recent coronavirus outbreak will certainly have negative effects on world tourism. Moreover, past experience demonstrates this, and in this article, we intend to analyse how the different epidemics that affected the globe after the 2000s influenced the tourism of the countries affected by these epidemics.

Thus, in 2000 there were about 200 deaths due to the epidemic with the H5N1 virus, the majority in Asia, which then reached Europe. The most affected countries were Indonesia, Vietnam, Thailand, China, but also Egypt. In Romania, the H5N1 virus appeared in 2005. In Turkey, the important tourist destination appeared in 2006. Both countries experienced decreases in the number of tourists and inseminated losses in terms of income from tourism. Also, Hungary, Czech Republic, Russia reported cases of H5N1, which in turn were economically affected. In 2001, foot-and-mouth disease or "cow disease" did not occur in the United Kingdom, and which only affected the cattle and was not transmissible to humans, causing great damage to tourism, but mainly to rural tourism in this country.

In November 2002, the first case of SARS (severe acute respiratory syndrome) appeared in China. The epidemic started in the Chinese province of Guangdong then spread throughout Southeast Asia, reaching Hong Kong, Taiwan, Vietnam and Singapore, and then spreading to 20 countries.

In 2012, another coronavirus, MERS, appeared in the Middle East and then expanded into several epidemic episodes.

As for COVID-19, it was reported in China, in Hubei Province, Wuhan City in December 2019, thus alerting the World Health Organization. In January 2020 it was identified as a new virus. To date, the virus has reached Europe, Italy being the European country with the most cases of infection and

deaths, but in many cases, it has also been reported by South Korea and Iran.
All these events had negative effects on the tourism market, one of the most developed markets globally and which has an important contribution in the formation of the gross domestic product worldwide (Popescu, 2015). Economic effects are important, considering that tourism is one of the most dynamic sectors of activity, which contributes to the creation of new jobs and to the economic development (Fintineru et al., 2014).
Tourism has benefited from all the advantages of technology, which has led to its development and to the increase of consumers' demands in this sector. The tourists of the 21st century are people who have access to information technology, who are dependent on communication, who want complex and individualized services, who are defenders of the environment, who are in search of new experiences, of quality, but at the same time of authenticity and original. This is possible in a world that is participating in a process of globalization that allows more and more people to travel, to visit, to explore. This is much easier in the conditions of the separation of the online booking services, in the conditions of the appearance of more and more low-cost airlines, now accessible to many travellers.
But it is precisely the fact that these trips are becoming more accessible, the fact that business has become international, contributing to the spread of epidemics with great ease and in a very short time.
And the tourist, being informed, is increasingly influenced by the decisions he makes to ensure his security, paying attention to events such as political instability, terrorism or the existence of epidemics.
Thus, in the field of tourism, globalization has contributed to the abolition of borders and the development of travel, but at the same time it has brought with it disadvantages that have negative effects on this sector (Dorobanțu et al., 2019).

MATERIALS AND METHODS

The objective of the present paper is to analyse how the occurrence of coronavirus (COVID - 19) at the beginning of 2020 will influence the tourist activity in Asia and Oceania, as well as in the other countries where the virus has spread, starting from the experiences of the last 20 years.
The working methods used are the review of the specialized literature, the empirical analysis of the information provided by the international databases, as well as the quantitative analysis which, through indicators such as the number of victims, the number of tourist arrivals, the value of the tourism receipts, etc. they will allow us to draw conclusions on how the events described will influence the tourism sector.
For the characterization of the dynamics of the studied phenomena, both absolute and fixed-base and chain-based indicators were used, as well as relative indicators, using the consecrated formulas (Danciulescu, 2020):

$$\Delta t_{/1} = y_t - y_1 \, ; \; \Delta t_{/t-1} = y_t - y_{t-1}$$

as well as:

$$I_{t/1} = \frac{yt}{y1}; \; I_{t/t-1} = \frac{yt}{yt-1},$$

where:
y1 - the value of the indicator in the reference period; yt - value of the indicator in period t;
yt-1 - the value of the indicator in period t-1.

RESULTS AND DISCUSSIONS

The analysis I made in this paper started from the way in which the epidemics of the 21st century influenced the tourist circulation and the incomes in tourism.
The study starts from the data referring to 1995, demonstrating that worldwide there have been increases in this sector of activity, both in terms of incomes obtained, as well as those regarding the number of tourists.
Thus, we watched how the SARS epidemic triggered in 2002 in China influenced the tourism of this country, as well as of the Asia-Oceania area.
It is noted that the most important tourist movement was registered in Europe, followed by America and Asia. In Europe, the increase was 34 percentage points in 2004 compared to 1995, in America by 16 percentage points, and in Asia by 79 percentage points.

Table 1. Evolution of the number of tourists in the period 1995-2004 (Million)

Year	1995	2000	2001	2002	2003	2004
Europe	309.30	384.10	383.70	394.60	396.60	414.40
Asia and Oceania	85.00	114.90	120.60	131.10	119.30	152.50
America	109.00	128.00	122.10	116.60	113.10	125.80
Africa	20.40	28.20	28.80	29.50	30.80	33.20
Middle East	14.30	35.20	25.00	29.20	30.00	35.40

Source: Own calculation

As for the receipts, they registered the same trend.

It is found that the first place is Europe, followed by America and Asia-Oceania.

Table 2. The evolution of the revenues from tourism in the period 1995-2004 (USD Billion)

Year	1995	2000	2001	2002	2003	2004
Europe	212.2	232.6	228.3	243.2	282.9	326.7
Asia and Oceania	82.0	89.1	91.8	100.4	94.9	125.0
America	98.5	131.0	120.1	113.8	114.1	131.7
Africa	8.5	10.7	11.6	12.1	15.5	18.33
Middle East	9.8	13.3	12.7	12.9	16.8	21.03

Source: Own calculation

Tourism revenue in Europe increased by 54 percentage points, in Asia and Oceania by 52 percentage points, in America by 34 percentage points, and in the Middle East by 114 percentage points.

In light of these increases, events related to the outbreak of epidemics have led to major imbalances.

Thus, SARS that appeared in China in 2002 and considered to be the first of the global epidemics of the 21st century, has negatively influenced tourism in the Asian area. According to the World Health Organization, the virus has reached 32 countries, leading to the death of 916 people and producing 8,422 illnesses. Of these, 5,327 cases, i.e. 63%, came from China.

As can be seen from Table 3, from 1995 to 2002 there has been a continuous increase in the number of arrivals in China. The increase from 2002 to the base year was about 84 percentage points. In 2003, the year in which SARS caused the epidemic, the decrease in the number of tourist arrivals compared to the previous year was 10 percentage points. And regarding the revenues from tourism, it can be seen that after a continuous increase in the analyzed period, there was a decrease of 3.1 million dollars in 2013.

What is noteworthy is the fact that although in 2000 mankind also faced the H5N1 epidemic, which again had the most victims in Asia, it did not have strong influence on the tourism activity in the analyzed area.

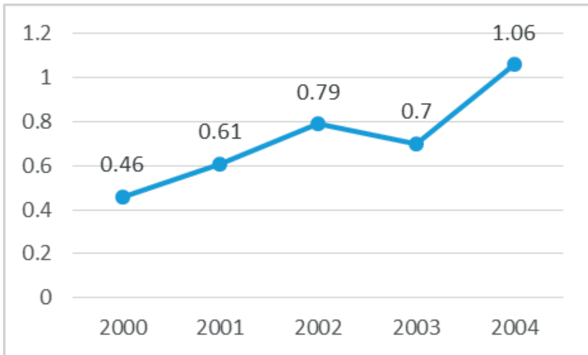

Figure 1. Evolution of the number of tourists in Cambodia.
Source: Own processing after (Elci, 2006)

One of the most affected countries was Cambodia, where the number of tourists did not decrease significantly (Figure 1), the losses being felt only in the production and marketing sectors.

Not the same can be said about how the H5N1 virus affected Romania's tourism in the Danube Delta, where the virus was discovered in a pelican species.

The data show how the number of tourists arriving in the Danube Delta fluctuated with the reporting of bird flu cases (Figure 2).

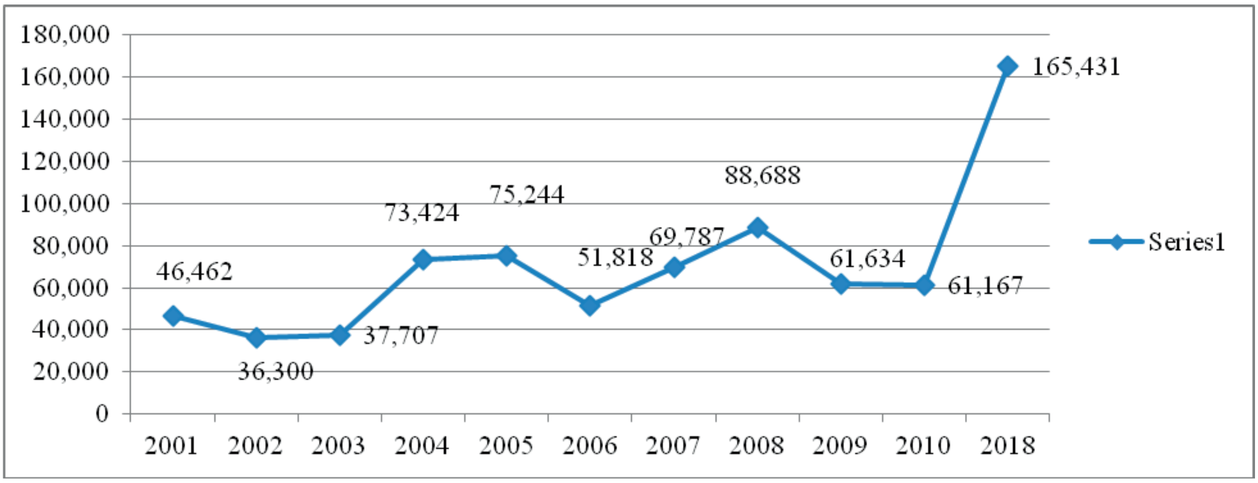

Figure 2. Arrivals situation in the tourist reception structures in the Danube Delta
Source: Own processing after (INS, 2019)

Next, we followed the evolution of annual visitor arrivals in China, because it is the country that constantly appears at the center of the epidemics, being the country that provides a large number of tourists to world tourism but also the country that receives tourists from all over the world.

Table 3. The situation of arrivals and incomes from tourism in China between 1995 and 2003

Year	Tourist arrivals		Tourism receipts	
	(thousand)	%	(millions $)	%
1995	20,034,00	100.0	8,733	100.0
1996	22,765.00	113.6	10,200	116.8
1997	23,770.00	118.6	12,074	138.2
1998	25,072.90	125.1	12,602	144.3
1999	27,046.60	135.0	14,099	161.4
2000	31,228.80	155.9	16,224	185.8
2001	33,166.70	165.5	17,792	203.7
2002	36,802.60	183.7	20,385	233.4
2003	32,970.50	164.6	17,406	199.3

Source: Own processing after (Lee et al., 2006)

Thus we find that during the analyzed period, there was an increase in the number of visitors until 2002. In 2003, their decrease is over 6 million visitors. Of these, foreign visitors represented between 11.2% in 1998 and 13.7% in 2002. The rest are represented by visitors from the Asian area (Hong Kong, Macao, Taiwan).
The number of people infected with MERS in 2012 was over 1,200 cases, the majority coming from Saudi Arabia, but then it reached other states, in the Asian area, South Korea being severely affected. According to the KTO, over 25,000 tourists have canceled their trips to South Korea, 85% of them coming from China, Hong Kong or Taiwan. For Saudi Arabia, it was estimated that religious tourism losses in the global economy were $ 16 billion.

Table 4. Evolution of Annual Visitor Arrivals in China (persons)

Year	Total	Foreigners
1995	46,386,511	5,886,716
1996	51,127,516	6,744,334
1997	57,587,923	7,428,006
1998	63,478,401	7,107,747
1999	72,795,594	8,432,296
2000	83,443,881	10,160,432
2001	89,012,924	11,226,384
2002	97,908,252	13,439,497
2003	91,662,100	11,402,900

Source: Own processing after (Lee et al., 2006).

Going forward, and going into 2020, although so far only estimates have appeared regarding how the coronavirus epidemic will affect China's economy, as well as other countries dependent on the Chinese economy, experts believe that in terms of tourism, the losses will be over $ 22 billion.
This will be due to the decrease in the number of Chinese tourists, who contributed 149.7 million worldwide trips in 2018, up 15% compared to 2017 and contributing to the development of tourism in areas of Thailand, Cambodia, Philippines, Macao or Hong Kong, but also in Europe, as well as in other countries of the world. According to the World Tourism Organization in 2018, the number of Chinese tourists reached almost 150 million, compared to 4.5 million in 2000. Chinese tourists spent $ 277.3 billion on vacations, which is $

1,850/tourist, which puts them in first place among tourists worldwide.
At the level of the entire Asian economy, losses are estimated to reach $ 115 billion in gross domestic product due to tourism.

The map by Johns Hopkins CSSE shows that on February 28, 2020, the number of coronavirus infections reached 83,867 cases, most of them in Asia and Oceania.

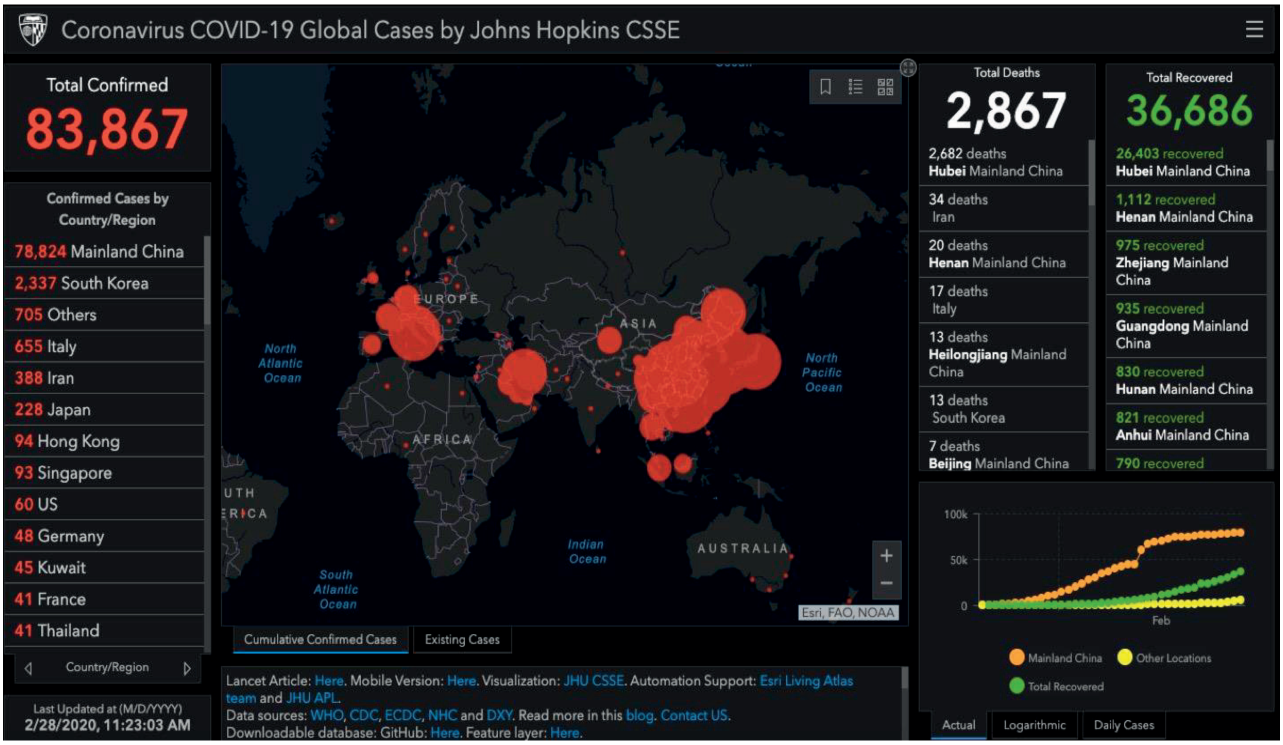

Figure 3. Situation of the areas carried out by COVID-19
Source: https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6, Accessed on 28.02.2020 (Gisanddata, 2020)

A study by UOB Global Economics & Markets Research on GDP forecast in Asia-Pacific countries shows that these drops will be between 0.5 and 1 percentage point, compared to initially projected values for 2020. Even though China is most affected by the epidemic with COVID-19, the losses of the other states are due to their dependence on Chinese tourists. Thus, in Singapore at the level of 2018, 20% of tourists came from China, in Malaysia they accounted for 11%, and in Thailand 28%.
From the published data it results that in Indonesia over 20,000 reservations of Chinese tourists were canceled, in Thailand it is estimated that their number will decrease by 2 million, representing the largest share of the total tourists from this country.
Also, in Macau, a favorite destination for gambling lovers, which ensures 70% of their total through Chinese tourists will incur significant losses.
Burma also states that the number of Chinese tourists has increased from 20% in 2018 to 38% in 2019, which at this time will influence the tourist economy.
In Europe, the number of Chinese tourists at the level of 2018 was 12.4 million, and the estimates for 2022 were 20.8 million. Under current conditions, their number is decreasing.

Table 5. Estimated GDP for 2020 in Asia

Country	2020 (baseline)	Estimated % point impact on baseline GDP
China	5.7	0.5-1.0
Hong Kong	1.2	0.7-1.5
Singapore	1.5	0.5-1.0
Taiwan	2.6	0.0-0.5
Thailand	2.8	0.5-1.0
Philippines	6.5	0.2-0.5
Malaysia	4.4	0.5-1.0
Indonesia	5.2	0.1-0.2
Vietnam	6.8	0.5-1.0

Source: Own processing after (Brodzicki, 2020)

Also from the scenarios that started to be made regarding the losses caused by the COVID epidemic - 19 and which are based on the experience of SARS and MERS, it results in a decrease of the revenues of the registered airlines not only in the affected areas, but globally. At the level of the entire industry, the decrease will be almost 29%.

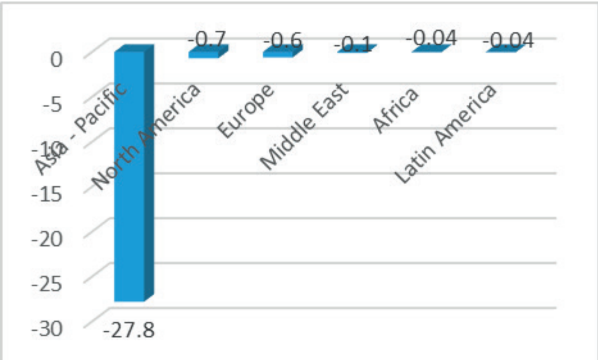

Figure 4. Decrease in airlines revenues in 2020 (estimate)
Source: Own processing after IATA
(IATA Economics, 2020)

And for Italy, the country strongly affected by the epidemic, the forecasts show decreases of GDP, given that it decreased in the last quarter of 2019.
This new crisis that appeared at the beginning of 2020 will contribute to the decrease of GDP, at least for the first period of the year, Italy being a country in which tourism contributes an important share to the formation of GDP.

CONCLUSIONS

Through this study we set out to show that both economic activity and tourism activity in the areas affected by the outbreak of epidemics are strongly influenced by them.
Although there is a high economic impact in the epicentre areas of their emergence, the fact that globalization has contributed to the regional and global integration of both tourism, trade, production, etc. causes the economic effects to be felt at the macroeconomic level.
The effects can have a long-term impact, being felt by the slowdown of economic growth, evidenced by the decrease of GDP in the affected regions due to the decrease of the production, the decrease of the investor confidence and of increasing the concern of the tourists.
It must be acknowledged that global tourism depends on Chinese tourists, and the decline of their number has important effects on the world economy.
Therefore, globalization, in addition to its advantages, also comes with risks that are amplified by its emergence and development. Their reduction, in the event of the occurrence of epidemics, can be achieved by implementing efficient public health policies, by correctly informing the population and by providing transparent and real-time information.
Regarding the forecasts made by specialists regarding the economic losses determined by the occurrence of COVID -19, they refer to an epidemic that will not exceed 6 months.
In the situation where this will extend the losses will be even higher, both in terms of economic losses, but also in terms of losses of human lives, which are ultimately the most important.

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PRODUCTION OF RABBIT MEAT WITH FUNCTIONAL PROPERTIES

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Abstract

The present work is based on the assumption that by supplementation thyme to defined concentration to feed of rabbits will improve the nutritional qualities of the meat and create opportunities for the development of safe products with high antioxidant activity, preserved nutritional value and easy digestion. This combination could protect against oxidative stress and increase the functional properties of the meat. To the feed of one experimental group was added 3% thyme (group T3) and to the other 5% thyme (group T5) at the expense of alfalfa hay (31.95% in control fodder). In the dispersion analysis we found that statistically significant differences were found only with respect to the weight of the liver which was lower in the groups fed with supplements of thyme compared to the control group, no significant differences were found with respect to the weight indicator. The incorporation of thyme into the fodder for fattening rabbits results in improved fatty acid composition in feed and meat, with the best results being achieved in rabbit breeding with the addition of 5% thyme leaves.

***Key words**: fodder, thyme, rabbit meat, fatty acids.*

INTRODUCTION

The increasing consumer demand for conducive to good health food has pushed the meat industry to develop new strategies to optimize the nutritional composition to improve the image of meat and meat products (Toldrá and Reig, 2011). A major goal of these strategies is the presence of a biologically active substance (BAS) in the meat or meat product to provide the desired effect over a reasonable period, depending on whether or not the BAS is assimilated by the organism or is present in a significant amount or not in the final product as well as give the desired effect with its absorption for a reasonable period. The achievement of this effect is scientifically established, nutrition claims and claims for health properties are detailed and regulated by the EU (Reg. EU 1924/2006; Reg. EU 432/2012).

Consumer's preferences for natural foods direct the scientific research into the potential of using natural antioxidants. It has been found that rosemary, green tea, ginger, marjoranam and thyme show a strong inhibitory effect on the lipid oxidation of meat products (Collignan and Montet, 1998; Kanner, 2007). Thyme and rosemary are widely used spices in the culinary treatment of various types of meat and in recipes for preparing sausages, pizza, spaghetti, etc. The composition of the extracts is well known and studied and they are classified as safe for use in foods. The essential components of essential thyme oil are the phenolic compounds thymol, carvacrol, and p- cimol, which have been found to inhibit most of the pathogenic microorganisms encountered in food of animal origin. In summary the problem of providing quality and safe meat products is current in Bulgaria and internationally scale. While many scientific teams are involved in the subject abroad, there is insufficient scientific research into the use of substances of natural origin as antioxidant stabilizers in meat products.

The possibilities for the introduction of biologically active substances from plant origin into the food technology as antioxidant stabilizers, especially in the preparation of raw meat products, have not been fully investigated. Required further research and an accurate assessment of the quantity and type of extracts which may be added to the foodstuff so as to achieve the desired concentration without deteriorating the organoleptic characteristics of the product (stronger or different odor and taste, color changes, etc., which would be unacceptable to the consumer). To achieve a proper balance between the antioxidant, flavoring and aromatizing action of plant extracts when used as additives in meat products, in-depth scientific and technological research is required. This analysis gives reason the efforts of the team to focus on searching for similar methods to improve the quality characteristics of meats in the composition of lyophilized foods with special uses.

The European Union initially restricted the use and then finally prohibited the use of antibiotics and other synthetic BAS as growth promoters in animal nutrition (Resurreccion, 2003) and the negative public opinion on the use of antibiotics, more and more scientific studies are devoted to natural alternatives (Laguerre et al., 2007; McAfee et al., 2010). The EU's decision stems from the fear that, despite the low dosing of antibiotics given for animal productivity or prophylactic purposes, may result in the formation of resistant strains of human pathogens that represent a real health risk to the population. In this regard, our main objective will be to evaluate the effect of thyme nutritional supplements (*Thymus serpyllum*) and the digestibility of these nutrients in rearing rabbits. Thyme is confirmed as a rich source of natural antioxidants and is an effective natural additive in the production of foods enriched with natural biologically active substances that are successfully applied in the meat (Stabler & Allen, 2004). Further research is needed to clarify the effects of thyme in fat deposition, bone development and mineralization. Changes in carcass fat are observed with thyme dietary supplements, but further studies are required to demonstrate whether and how these supplements can affect lipid metabolism. Therefore, our study is focused on the use of these additives to retention and protection against oxidative stress in the meat. According to the literature, a 5% of thyme supplement (*Thymus serpyllum*) is effective for increasing gamma linolenic acid (GLA) (FAO, 1997; FAO/WHO, 1991). Thyme, on the other hand, improves the oxidative stability of raw and lyophilized meat, but not of boiled meat (Peiretti and Meineri, 2011; Sikorski and Kolodziejska, 1986).

The present work is based on the assumption that by supplementation thyme to defined concentration to feed of rabbits will improve the nutritional qualities of the meat and create opportunities for the development of safe products with high antioxidant activity, preserved nutritional value and easy digestion.

MATERIALS AND METHODS

Experimental Animals

The experiment was conducted in the experimental rabbit farm of the Institute of Animal Science - Kostinbrod. Participation took 30 rabbits. The experiment started with 55 days old rabbits. The experience is of long duration from 7 weeks, to an average live weight above 2100 g. The rabbits were divided into three groups of 10 animals: one control and two experimentals. The animals are fed with whole-grained granular mixtures.

Feeding of experimental animals

The feed used in the experiment was prepared at the Agricultural Institute in Stara Zagora by recipe for combined fodder a fattening of rabbits 53- 3- 6 on August 02, 2017 (Protein-14.26%, Fibber-13.43%, Fat-1.83%, Energy-1979.62 Kcal / kg). Three percent thyme (group T3) and 5% thyme (group T5) were added to the feed of one experimental group at the expense of alfalfa hay (31.95% in control fodder). In the first two weeks of the experiment, rabbits were fed restrictively. They received 100 grams fodder of rabbit per day. After this period, the animals of the three groups were fed freely. They took water through nipple drinkers.

Weight development

During the experiment, rabbit weight data were collected weekly after approximately 18 hours of starvation.

Slaughter analysis

A total of 6 rabbits were slaughtered in each group (18 in total). The following data were taken during the slaughter: live weight before slaughter, body weight after slaughter, and weights of the individual organs.

Statistical processing and analysis of the results obtained

All results are presented as mean ± SD (standard deviation) at 6 replicates for each sample. The results are considered reliable at p

< 0.05. All statistical analyzes are performed with Excel 2013.

Biochemical Studies

Fatty acid analysis of meat performed - the total lipid extraction was performed by Bligh and Dyer (1959) with chloroform and methanol in a ratio of 1: 2. The methyl esters of fatty acids (FAME) were analyzed using a Shimadzu-2010 gas chromatograph (Kyoto, Japan). The assay is performed with a CP7420 capillary column (100 m x 0.25 mm i.d., 0.2 m, Varian Inc., Palo Alto, CA), with carrier gas-hydrogen and make-up gas-nitrogen. A five-step gas chromatographic oven program has been used.

RESULTS AND DISCUSSIONS

Six rabbits from each group were slaughtered (18 in total). On the basis of the slaughter analysis of the animals, it was established weight change of the rabbits in the different groups relative to the start of the measurements. At the end of the period, we reported an increase of about 100% in the control groups fed with 3% thyme fodder in one and 5% thyme in the other. The animals in the third group had the highest weight gain compared to the beginning of the measurements and the other two groups (Table 1).

Table 1. Relatively variation on rabbit weight by different regime on nutrition toward beginning of measurement, (%)

Regime of nutrition	Beginning	1 week	2 week	3 week	4 week	5 week	6 week	7 week
Control - usual feed - average value	100	102.92	112.34	125.52	140.18	151.18	166.31	177.27
Feed with 3% *Thymus serpyllum* - average value	100	103.62	119.78	138.75	154.46	163.15	177.14	191.13
Feed with 5% *Thymus serpyllum* - average value	100	107.65	122.97	141.52	159.86	176.98	189.95	202.22

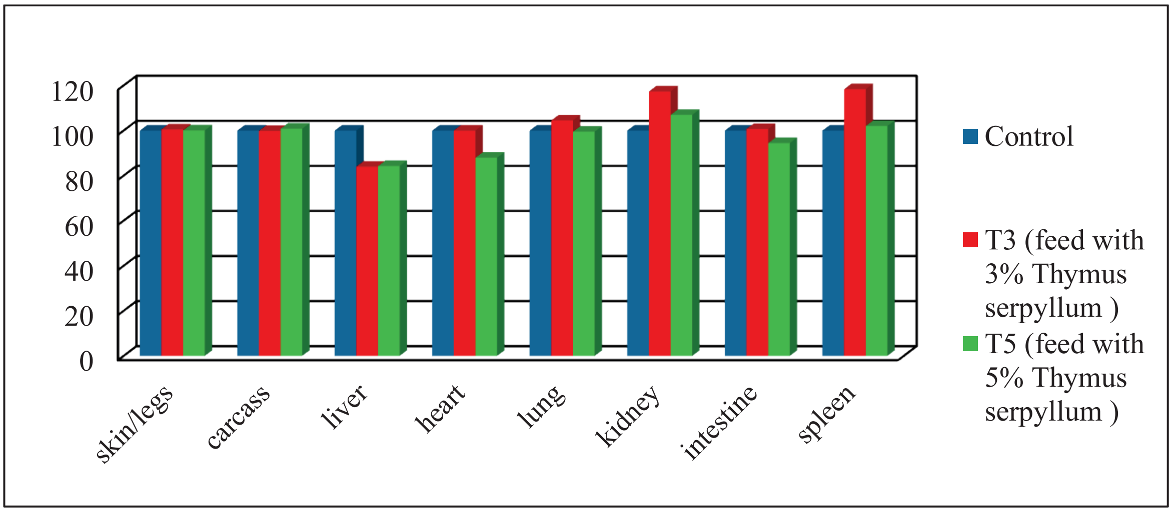

Figure 1. Weight of anatomic organs proportion on rabbits feeded with suplement *Thymus serpullum* (3 and 5%) relation to control group

It can be seen from Figure 1 that with respect to the weight of carcass meat, skin, legs, lung and intestines we have a small differences in the control group. We found lower liver weight in both variants, less heart in group T5 and increased spleen and kidney in group T3. Subsequent dispersal analysis will answer the question of how far these differences are substantial and statistically significant.

Deviation from normal organ weights in rabbits is usually a symptom of some common diseases such as coccidiosis (enlarged liver), pasteurellosis (enlarged spleen) etc.

When done dispersion analysis (Table 2) we have established that statistically significant differences were found only with respect to the weight of the liver, which is lower in the groups fed with addition of thyme compared to the control group.

Fatty acids in feed for fattening rabbits

The fatty acid composition of the feed for fattening rabbits has a saturated fatty acid (SFA) content of 40.39 g/100 g fat, monounsaturated fatty acids (MUFA)-33.41 g/100 g fat, polyunsaturated fatty acids (PUFA)-10.91 g/100 g fat, trans isomers of linoleic acid-0.67 g/100 g fat, cis isomers-3.81 g/100 g fat, omega-3 fatty acids-0.68 g/100 g fat and omega-6 fatty acids-9.65 g/100 g fat. The using of supplement from 3 and 5% thyme leaves in the fodder leads to an improvement in its fatty acid composition with respect to the content of the cis isomers of linoleic acid, respectively to 7.43 and 9.90 g/100 g fat (2 and 2.6 times the output feed), saturated and polyunsaturated fatty acids increase as follows from 12 to 13 g/100 g fat for SFA and from 3 to 7 g/100 g fat for PUFA, at the expense for MUFAs, which decrease by 8 and 9 g/100 g fat. The trans isomers of linoleic acid vary in a narrow range. The total omega-3 fatty acid content increases threefold with supplement with 3% thyme and four times with 5%. The omega-6 fatty acid content is increased to 10.82 g/100 g fat with 3% thyme added and up to 13.98% with 5% thyme supplement. The ratio between the biologically active omega-6 and omega-3 fatty acids decreases by 2.5 times as a result of their change due to the addition of thyme and the optimal ratio of 5.55 to 5% of the additive. Application of thyme as a feed additive improves the content of branched fatty acids resulting from microbiological activity and decreases by 1.6 times at 3% and 3 times at 5%. This is due to its antimicrobial and antioxidant properties (Table 3).

Table 2. Dispersion analysis on weight difference on anatomic organs in rabbit groups in dependence of regime on nutrition

Organ	Factor on investigation F_{kp} 0.05=4.96; F_{kp} 0.01=10.04; F_{kp} 0.001= 21.04					
	Between control and feed with 3% *Thymus serpyllum*		Between control and fee with 5% *Thymus serpyllum*		Between feed with 3% and 5% *Thymus serpyllum*	
	$F_{оп}$	Rank of proof	$F_{оп}$	Rank of proof	$F_{оп}$	Rank of proof
Skin/legs	0.014	-	0.001	-	0.008	-
Carcass	0.009	-	0.599	-	0.514	-
Liver	10.612	**	5.520	*	0.002	-
Heart	0.000	-	1.206	-	2.830	-
Lung	0.238	-	0.002	-	0.123	-
Kidney	2.332	-	1.053	-	0.747	-
Intestine	0.012	-	0.607	-	0.931	-
Spleen	2.022	-	0.057	-	1.257	-

P< 0.001 - ***; P< 0.01 - ** ; P< 0.05 - *

The major constituents of saturated fatty acids are lauric, palmitic and stearic acid. Lauric acid (C12:0) increased from 9.95 to 13.51 g/100 g fat with a 3% addition and up to 12.14 g/100 g fat in the addition of 5%. The palmitic acid (C-16:0) was found to be twice reduced by the addition of 5% thyme. The stearic acid (C-18:0) increases twice with addition of 3% thyme and 3 times with 5% thyme. Palmitelaidic acid (C-16:19tr) in the analyzed fodder is 19.24 g/100g fat, but the edition of 3% of thyme were reduced her by 2.5 times and with 5% of thyme by 4 times. Palmitoleic acid (C-16:1n7) has a concentration of 1.44 g/100 g fat, but due to the incorporation of thyme, its content decreases to traces in the analyzed feed. Oleic acid (C-18:1c9) in fodder for fattening of rabbits is 3.45 g/100 g fat and increases twice with 3% thyme and 2.5 times with 5% thyme addition. The linoleic acid increases from 1.55 g/100 g fat in feed to 2.20 g/100 g fat in the addition of 3% thyme and 2.27 g/100 g fat in 5% thyme. In the linoleic acid were increased with 5% thyme introduced from 3.15 in the raw material to 4.46 g/100 g fat in the additive used. As a result of the incorporation of thyme in the feed for fattening, it is found that CLA9c,11c and CLA9t,11t increase from 0.60 to 1.04 and 0.08 to 0.37 g/100 g fat. These isomers are characteristic of plant species and the probable variation is due to a higher concentration in the thyme leaves. Changes occur and in content of omega-3 and omega-6 fatty acids. An increase in the content of dihomo gamma linolenic acid (C-20:3n6) was found as follows in 3% thyme-6 times and 5%-7 times. Arachidonic acid (C-20:4n6) increased significantly with the addition of thyme and the highest content from 1.21 g/100 g fat was found at 5%. Eicosapentaenoic acid (C-20:5n3), followed by the change in arachidonic fatty acid (Table 3).

Table 3. Fatty acids (g/100 g fat) in fodder for fattening of rabbits

Fatty acids	Control	3% *Thymus serpyllum*	5% *Thymus serpyllum*
C-12:0	9.95	13.51	12.14
C-16:0	10.49	11.06	6.95
C-18.0	6.69	16.93	20.88
C-16:19tr	19.24	7.84	5.05
C-16:1n7	1.44	0.01	0.00
C-18:1c9	3.45	6.63	8.60
C-18:2c9,12/19:0	1.55	2.20	2.27
gC-18:3n6	3.15	2.04	4.46
aC-18:3n3	0.01	0.45	0.54
CLA9c,11c	0.60	0.84	1.04
CLA9t,11t	0.08	0.23	0.37
C-20:3n6	0.04	0.25	0.28
C-20:4n6	0.03	0.70	1.21
C-20:5n3	0.07	0.16	0.39
∑CLA	0.67	1.06	1.41
∑C-18:1Trans-FA	0.64	0.50	0.39
∑ C-18:1Cis-FA	3.81	7.43	9.90
SFA	40.39	52.22	53.18
MUFA	33.41	25.27	24.52
PUFA	10.91	13.51	17.48
∑n-3	0.68	1.88	2.52
∑n-6	9.65	10.82	13.98
∑n-6/Σn-3	14.16	5.76	5.55
Branched FA	15.29	9.00	4.83

Fatty acids in rabbit leg meat rearing with fodder supplement by thyme

The fatty acid composition of analyses rabbit meat has a 75.05 g/100 g fat saturated fatty acid content (SFA) in the control and reduced to 70.56 g/100 g fat in meat obtained with 5% thyme feed. Monounsaturated fatty acids (MUFA) - in control group is 12.88 and do not change when used supplement of 3% thyme in the diet, while in 5% thyme addition increases to 18.08 g/100 g fat. Polyunsaturated fatty acids (PUFA) in the control group were the lowest - 5.24 g/100 g fat and increased to 6.63 g/100 g fat in 3% thyme and up to 7.08 g/100 g fat in 5% thyme. Trans isomers of linoleic acid in the control group meat are 2.83 g/100g fat and increase to 3.92 g/100 g fat in 3% thyme and 5.63 g/100 g fat in 5% thyme in the diet. The cis isomers in the analyzed meats are 3.28 g/100 g fat, reduced by 3% of thyme to 1.87 g/100 g fat and increased to 5.18 g/100 g fat in 5% thyme supplement of fodder. Omega-3 fatty acids increased relative to the control group as follows from 2.63 g/100 g fat in control group to 3.46 g/100 g fat in 3% thyme and up to 4.24 g/100 g fat in 5% thyme rearing group. Omega-6 fatty acids do not undergo substantial changes in the overall content. The ratio between the biologically active omega-6 and omega-3 fatty acids decreases from 1.16 g/100 g fat in the control group to 0.83 g/100 g fat when 5% thyme is added in feed. Application of the thyme as a feed additive results in a reduction in the branched fatty acid content from 6.84 g/100 g fat in the control group to 4.28 g/100 g fat in 5% thyme supplement in fodder (Table 4).

Caprylic acid (C-10:0) in the analyzed meats increased from 0.46 g/100 g fat in control group to 0.51 g/100 g fat in 3% thyme and 0.74 g/100 g fat in 5% thyme addition. The lauric fatty acid (C-12:0) increases at 3% thyme relative to the control group from 2.74 to 5.00 g/100 g fat and in 5% of thyme group to 3.94 g/100 g fat.

Table 4. Fatty acid (g/100 g fat) in rabbit leg meat rearing with fodder supplement by *Thymus serpyllum*

Fatty acids	Control	3% *Thymus serpyllum*	5% *Thymus serpyllum*
C-12:0	2.74	5.00	3.94
C-14:0	9.54	8.69	5.91
C-16:0	17.11	24.25	20.82
C-18.0	41.01	34.16	34.90
C-16:19tr	1.51	0.77	0.88
C-18:1c9/C-18:1t12/13/	2.62	1.41	4.50
C-18:2c9,12/19:0	0.55	0.30	0.39
gC-18:3n6	0.63	0.37	0.28
aC-18:3n3	0.41	0.27	0.43
C-20:3n6	0.58	0.76	0.71
C-20:4n6	0.10	0.30	0.29
C-20:5n3	0.26	0.38	0.41
C-22:5n3	0.69	0.85	1.12
∑C-18:1Trans-FA	2.83	3.92	5.63
∑ C-18:1Cis-FA	3.28	1.87	5.18
SFA	75.05	77.21	70.56
MUFA	12.88	12.60	18.08
PUFA	5.24	6.63	7.08
∑n-3	2.63	3.46	4.24
∑n-6	3.05	3.60	3.51
∑n-6/Σn-3	1.16	1.04	0.83
Branched FA	6.84	3.56	4.28

Myristic acid (C14:0) decreases from 9.54 g/100g fat in control group to 8.69 g/100 g fat in 3% thyme group and to 5.91 g/100 g fat when 5% thyme is added to the diet. The palmitic acid (C-16:0) have a higher content between control group and group with supplement of 3% thyme in the diet from 17.11 to 24.25 g/100 g fat were established. Stearic acid (C-18:0) decreases from 41.01 g/100 g fat in control group to 34.16 g/100 g fat in T3 group and is retained in T5 group-34.90 g/100 g fat (Table 4). Palmitelaidic acid (C-16:19tr) in the analyzed meat decreased twice in the case of supplement with thyme of fodder. Oleic acid (C-18:1c9) increased in the meat of rabbits fed 5% thyme twice relative to the control group. Linoleic acid decreases from 0.55 g/100 g fat in the control group meat to 0.30 g/100 g fat with 3% thyme and 0.39 g/100 g fat with 5% thyme. Gamma linolenic acid decreases twice when 3 and 5% of thyme is added to the fodder relative to control. Alpha linolenic is lower in content using 3% thyme while 5% did not undergo changes compared to the control group of meats. Dihomo gamma linolenic acid (C-20:3n6) increases with 3 and 5% thyme incorporation by 1.3 times the control group. Arachidonic acid (C-20:4n6) increased threefold in the meat by the supplementation with 3 and 5% thyme in the rabbit diet versus the control group fed with fodder. Eicosapentaenoic acid (C-20:5n3) increased from 0.26 g/100 g fat in the meat of control group to 0.38 g/100 g fat in the 3% thyme fed group and to 0.41 g/100 g fat in 5% thyme fed group. Docosahexaenoic acid (C-22:5n3) increases from 0.65 g/100 g fat in the meat control group to 0.85 g/100 g fat in the 3% thyme fed group and up to 1.12 g/100 g fat in 5% thyme fed group.

De Andrade et al. (2018), applying a variety of rabbit rearing diets, found a lauric acid content from 3.84 to 10.03%, myristic acid from 2.55 to 2.88%, palmitic acid from 16.48 to 26.56%, stearic from 6.12 to 8.79%, C-16:1n7 from 2.30 to 4.72%, oleic from 22.93 to 28.29%, linoleic from 17.54 to 30.22%, linolenic from 1.1 to 2.4%, saturated fatty acids from 32.4 to 49.2%, MUFA from 25.1 to 35.45 and PUFA from 25.6 to 32.1%. Mattioli et al. (2017) in a study of oregano supplement, vitamin E and probiotic in rabbit nutrition and rabbit loan and leg analysis, found higher values than ours for stearic fatty acid and polyunsaturated fatty acids and lower for myristic, palmitic, oleic, SFA and MUFA. Yonkova et al. (2017) were found a higher content of saturated and monounsaturated fatty acids and a lower content of polyunsaturated fatty acids compared to those obtained in our study and an identical ratio of omega-6 to omega-3 fatty acids. Identical results for the fatty acid composition of rabbit meat were obtained by Rasinska et al. (2018).

CONCLUSIONS

In regard to the weight of the carcass meat, skin, legs, lungs and intestines, get small differences across the control group. We found lower liver weight in both variants, less heart in group T5 and increased spleen and kidney in group T3. In the dispersion analysis we found that statistically significant differences were obtained only with respect to the weight of the liver, which was lower in the groups fed with added thyme compared to the control group. The results of the dispersion analysis show the existence of statistically significant differences in the weight of the test specimens between the thyme-supplemented and control variants, while in the feed diet supplemented with 5% thyme, the differences were the highest confidence level for almost the entire study period. Between the two variants fed with 3 and 5% thyme almost no significant differences were found with respect to the weight indicator. The incorporation of thyme into the fodder for fattening of rabbits results in an improvement of the fatty acid composition in feed and meat, with the best results being achieved by rearing rabbits fed with fodder enriched with 5% thyme leaves. The supplementation by thyme in the rabbit feed leads to a decrease in branched fatty acids, which is an indicator of improving the quality of meat and increasing the biologically active omega-3 fatty acids. Best results are achieved using 5% thyme leaves. This leads to improving fatty acid content of meat, due to the increase in biologically active fatty acids-trans and cis isomers of oleic acid, omega-3 and omega-6 fatty acids. Best results have been achieved in rearing rabbits fed with 5% fortified fodder with thyme leaves.

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PRELIMINARY STUDY ON EVALUATION OF THE INTRAOPERATIVE BACTERIAL CONTAMINATION OF THE SURGICAL WOUND IN SMALL ANIMALS

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Abstract

The intraoperative contamination of the surgical wound is a veterinary issue as it is closely related to surgical practices. It can originate from the patient's endogenous flora, from the breakage of the sterility barrier or from the surrounding environment. This study aimed to assess the incidence, associated factors and bacteria isolated from 64 surgical wounds in dogs and cats that underwent surgery. It was also evaluated the influence of two different types of incisional surgical drapes, with and without Betadine®, for the control and the protection of the surgical site. It was found that, at the end of the surgery, 54% of the surgical wounds were contaminated and it was also demonstrated that adhesive surgical drapes play a crucial role in reducing the percentage of contamination. The most frequently isolated bacteria were: Staphylococcus spp., Gram-negative, oxidases-positive and bacilli. Therefore, according to these results, a thorough analysis of the risk factors and the development of innovative techniques and instruments are necessary to maintain the intraoperative bacterial load at sub-pathogenic levels.

***Key words**: adhesive surgical drapes, intraoperative contamination, surgical wound.*

INTRODUCTION

Maintaining conditions of surgical sterility, combined with a preoperative protocol and the use of optimal surgical practices, is of paramount importance to prevent potential contamination of the surgical field.
Contamination of the surgical field refers to the presence, at the end of the surgical procedure, of microorganisms capable of altering healing processes. If the population of microorganisms is protected, the contaminated wound will become infected (Ahrendt et al., 1996; Cochrane, 2010).
Surgical site infection (SSI), could be a consequence of contamination and lead to postoperative complications of different severity that require extended management of the surgical wound with an increase of the cost by the owner (Nelson, 2011; Nishikawa et al., 2008).
Bacteria isolated at the end of surgery can be endogenous or exogenous (Edmiston et al., 2005; Oguz et al., 2017).
Endogenous bacteria include microflora residing in the superficial skin or in the deeper layers (subcutaneous, muscle or internal organs) and include aerobic and anaerobic bacteria, Gram-positive (e.g. *Staphylococcus* spp.), and Gram-negative, depending on the surgical area. Bacteria of exogenous nature can contaminate the surgical field from the surrounding environment (aerosol) or as a consequence of sterility barrier breakage and are mainly aerobic, especially Gram-positive (e.g. *Staphylococcus* spp. and *Streptococcus* spp.) (Tschudin-Sutter et al., 2012; van Ek et al., 1986; Bucur et al., 2015; Zapryanova et al., 2013).
In addition to the species mentioned above, it is sometimes possible to find the presence of *Micrococcus* spp., *Corynebacterium* spp. and *Bacillus* spp. as well as Gram-negative species such as *Stenotrophomonas maltophila*, *Burkholderia cepacia* and *Pseudomonas* spp. (Geiger et al., 2001).
Microbial contamination of the surgical site is, therefore, a necessary but not the only

precursor to the development of infections, underlining that not all contaminated wounds result in the subsequent infection (Nishikawa et al., 2008).
The predisposing factors for contamination are related to the patients themselves (age, concomitant pathologies, endocrines disease) and their management during the pre and intraoperative phases (Graf & Vonberg, 2014; Haridas & Malangoni, 2008; Malone et al., 2002; Mangram et al., 1999).
In the preoperative phases, the use of trichotomy, scrub and antimicrobial prophylaxis protocol aims to reduce the microbial load and keep it at sub-pathogenic levels for the duration of the surgery (Classen et al., 1992; Fossum, 2013; Junker et al., 2012; Neihaus et al., 2011).
In the intraoperative phases, however, it is necessary to check temperature, oxygenation and any complications - such as hypotension or bleeding - that affect the microcirculation of the wound (Eugster et al., 2004; Greif et al., 2000; Pokrywka & Byers, 2013).
Factors not related to the patient that can influence the degree of contamination are the duration of the surgery, anaesthesia and the operating room (OR) ventilation (Edmiston et al., 2005; Leong et al., 2006).
To reduce and prevent bacterial translocation, it is furthermore possible to use adhesive surgical drapes, whether or not impregnated with betadine, which form a semi-occlusive physical and chemical barrier (Owen et al., 2009; Yoshimura et al., 2003).

MATERIALS AND METHODS

The data for this study were systematically collected from dogs and cats undergoing a surgical procedure at the University Veterinary Teaching Hospital (OVUD) of the University of Perugia between March 2019 and September 2019 for a total of 51 animals (40 dogs, 11 cats) and 68 surgical wounds.
The same induction protocol and an extensive trichotomy of the area undergoing surgery were carried out in all the subjects by using shearing machines in the preparation room before carrying the animal to the operating room.
A standard antimicrobial prophylaxis protocol was used and involved the administration of cefazolin (30 mg/kg intravenous [IV]) 30 min before the incision and every 90 min during surgery. The same aseptic preparation protocol was used for all patients: the animal's skin was cleaned, using sterile gloves, with the alternating application of 10% iodopovidone (Betadine®) and alcohol, completing the procedure with an iodine spray solution. The preparation of the surgeon and assistants was performed according to the protocol and asepsis standards.
The contamination of the wound was assessed through two samples: the first one was collected at the level of the presumed surgical access immediately before the incision, using a dry sterile swab, with a vigorous rubbing for 5-10 seconds alternating clockwise and anticlockwise movements. The second sample was collected immediately before the completion of the cutaneous suture. In addition to the swabbing, two other samples were taken by fine-needle aspiration and skin biopsy (Figures 1-3).
A sterile syringe was used for the needle-aspiration with 2-3 aspiration performed at the margins of the surgical wound.
Skin biopsy was sampled by resecting of approximately 1 mm of skin from the wound’s margin and was placed in a sterile tube.

Figure 1. Preoperative swab

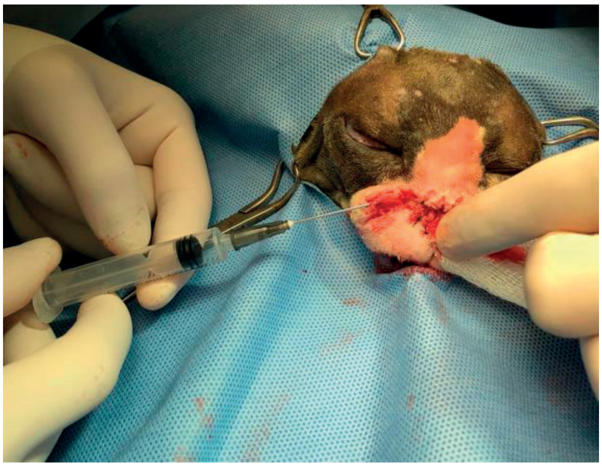

Figure 2. Postoperative fine-needle aspiration

Figure 3. Postoperative skin biopsy

The samples, adequately sealed and uniquely identified, were subject to bacteriological examination within 24 h.
All the samples were firstly placed in 1 ml of TSB (Tryptone Soya Broth) and incubated at 37°C for 24 h. Subsequently, the samples were streaked onto Blood Agar, Mannitol Salt Agar, MacConkey Agar (Liofilchem®), and then incubated aerobically at 37°C for 24-48 h.
The isolated colonies were evaluated based on morphological, dyeing and characteristics. Lastly, they were identified using miniaturised biochemical systems (API- Biomerieux®) in accordance with work protocols provided by the manufacturer.

Statistical Methods

The statistical analysis initially envisaged the calculation, using descriptive statistics, of mean and median for continuous data and frequency for categorical data. Subsequently, the $\chi 2$ test with Yates correction was used, with two-way tables, to compare all the categorical variables considering the p-value < .05 and < .10. For results within this range, logistic regression was performed to determine the Odds ratio of the potential wound contamination factors.

RESULTS AND DISCUSSIONS

Factors related to the patient (species, sex, age, weight) and to the surgical procedure (the type of surgery, duration of surgery and anaesthesia, use of surgical adhesive drape and its possible misplacement at the end of the procedure, complications during surgery) were analysed for each case included in this study.
51 animals were sampled between dogs (n = 40; 78.48%) and cats (n = 11; 21.52%) for a total of 68 surgical wounds (dog n = 52; cat n = 16).
Those cases in which two or more surgical accesses were foreseen for the same animal were analysed as two distinct cases.
Out of the 68 surgical wounds sampled, four were excluded from the study because a positive result was detected at the first post-scrub sampling due to an emergency (n = 1) or an incorrectly performed scrub (n = 3).
Table 1 shows the data collected concerning animals and surgery.
The degree of contamination found in the 64 surgical wounds analysed was 54% by assessing the positivity to at least one of the diagnostic tools used. It was also highlighted that the swab, despite being the most widely used tool in clinical practice to assess possible contamination, is less sensitive (11%) than fine-needle aspiration (32%) and skin biopsy (50%). (Parikh et al., 2007; Seidel & Bunse, 2017). A total of 29 cases had a negative postoperative swab, needle aspiration and skin biopsy (46%).

Table 1. Distribution of Patients and Intervention Characteristics (Quantitative)
Median (Average)

Age of dogs in years	3 (5.0)
Age of cats in years	2 (2.2)
Weight of dogs in kg	27 (27.5)
Weight of cats in kg	3.3 (3.3)
Duration of surgical intervention of dogs in minutes	100 (106)
Duration of surgical intervention of cats in minutes	113 (136)
Duration of anaesthesia of dogs in minutes	187.5 (180)
Duration of anaesthesia of cats in minutes	140 (143)

High contamination at the end of the surgery is not always associated with infection as the defensive mechanisms are able to ensure normal wound healing even in the presence of bacteria (Nishikawa et al., 2008).
The increased sensitivity of the skin biopsy may be due to the type of sampling as more material need to be taken.
The major issue associated with the use of skin biopsy is related to the difficulties on suturing and to the consequence cosmetic results: however, this problem was never encountered within this study because the skin sample was minimal (2 x 1 mm) and did not affect a conventional suture pattern.
The most isolated species, as shown in Table 2, belong to the genus *Staphylococcus*, mainly coagulase-negative staphylococci (28%), such *as Stapylococcus xylosus, S. chromogenes, S. lentus, S. saprophyticus, S. epidermidis and S. simulans* (Hsieh et al., 2014; Tschudin-Sutter et al., 2012).
The specie most frequently isolated was *Staphylococcus pseudintermedius*, found in 7 surgical wounds (10%). Other bacteria isolated include Gram-negative, oxidase-positive, like *Pseudomonas aeruginosa, Strenotrophomonas maltophila, Burkholderia cepacia* and *Pasteurella* spp., *Streptococcus canis,* which is not commonly found in the skin, is very often associated with infectious processes.

Table 2. Bacteria isolated from post-operative samples

Pathogen	Swab	Fine - needle aspiration	Skin biopsy
Coagulase-negative staphylococci	5	17	23
Staphylococcus pseudintermedius	3	6	6
Staphylococcus aureus	-	1	1
Bacillus spp.	-	1	2
Gram –, oxidase +	-	1	7
Escherichia coli	-	1	1
Streptococcus canis	-	-	1

Lastly, although with very low frequencies, populations of bacilli (*Bacillus* spp.) and in one case of *Staphylococcus aureus* were also found.Associating the presence of certain bacteria to the surgical site infection has not been demonstrated yet due to the amount of postoperative variables that can come into play, while it is possible to analyse isolated species for pathogenetic characteristics (Andrade et al., 2016).
Coagulase-negative staphylococci, as well as the different species of bacilli, are in most cases considered as minor contaminants, with little or no pathogenetic role: they are rarely protagonists and isolated from outbreaks of infection. Different considerations can be made for *Pseudomonas* spp., *Stenotrophomonas maltophila* and *Burkholderia cepacia* which, besides presenting an innate resistance to antibiotics, are very often associated with outbreaks of infection, altered healing processes and lung problems (Geiger et al., 2001).
Staphylococcus pseudintermedius, *E. coli* and *Staphylococcus aureus*, on the other hand, are the most frequently isolated bacteria from infectious outbreaks (Mangram et al., 1999; Owen et al., 2009).
For this reason, although it is challenging to associate and assess, from a clinical point of view, the presence of certain bacteria at the end of the surgical procedure and subsequent inflammatory/infectious phenomena, the presence of these species should be a warning sign for the monitoring and prophylaxis of the wounds themselves.
Although their use is controversial, this study showed a statistically significant reduction (OR = 3.327; CI = 95%; P = 0.028) in the frequency of contamination linked to the use of the surgical adhesive drape, with or without betadine. It was also shown that the betadine drape leads to a 3.6-fold reduction in contamination (OR = 3.6735; CI = 90%; P = 0.0501).
The results obtained with the use of betadine surgical drapes showed how combining both physical and chemical protection ensures a cleaner surgical procedure, as demonstrated in other scientific studies (Fairclough et al., 1986; Yoshimura et al., 2003).
A reduction in the effectiveness of the adhesive drape was observed when at the end of the surgery, a shift from the initial position had been reported (Figures 4, 5).

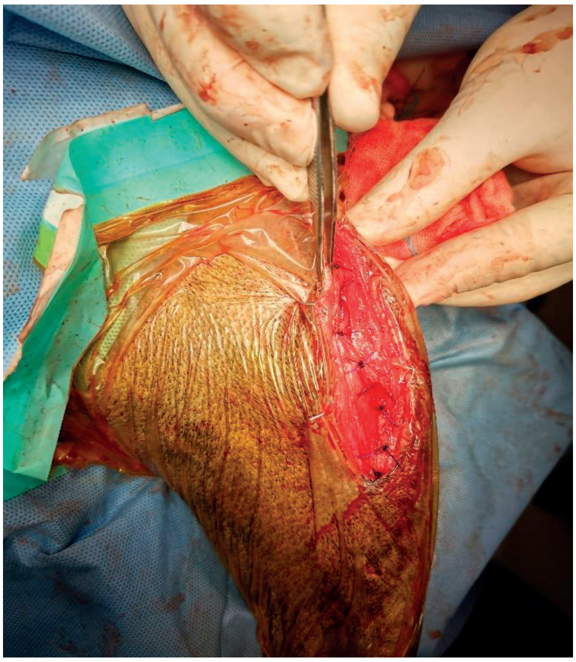

Figure 4. The adhesive drape is closely attached to the skin

Figure 5. The adhesive drape turns out to be moved in several places

In the case of a misplaced adhesive betadine drape, the percentage of contamination was 60% compared to 33% of non-displaced. In the case of a misplaced adhesive drape, the positivity after misplacement was 54% compared to 36% of non-displaced (Table 3; ASDB = Adhesive surgical drape with betadine; ASBWB = Adhesive surgical drape without betadine).

The different degrees of contamination obtained with the various types of surgical drapes also lead to commenting on the aetiology of bacterial contamination that can originate from: the patients themselves, the surgeon (failure of asepsis barriers) or from the surrounding environment. The use of adhesive drape is considered a physical barrier, reducing the skin surface exposed to the external environment; nevertheless, the betadine drape is capable of reducing the growth of the microbial flora keeping it at sub-pathogenic levels.

Table 3. Positive samples with each surgical drape

	ADSB	ADSWB	No Film
Dogs (%)	11 (22%)	21 (40%)	20 (38%)
Cats (%)	6 (37.5%)	2 (12.5%)	8 (50%)
Postoperative swab + (%)	0/17 (0%)	3/22 (13%)	4/25 (16%)
Postoperative fine-needle aspiration + (%)	5/17 (30%)	5/22 (22%)	11/25 (22%)
Postoperative skin biopsy + (%)	6/17 (35%)	9/22 (40%)	17/25 (68%)

In order to approximately evaluate the influence of the environmental, it was considered the number of working staff present in the operating room (average = 8). This high number was due to the fact that being a primarily didactic structure, many students are attending the surgery or anaesthesia.

No statistically significant associations were found between the degree of contamination and variables such as species (cat = 62.5%; dogs = 52%), sex (male = 59%; female = 50%), age (< 4 years = 59%; > 4 years = 41%) and weight (< 10 kg = 60%; > 10 kg = 54%).The duration of surgery, anesthesia and the hypothermia are considered to be the most involved factors in the development of contamination and infection: any clean procedure lasting more than 90 min is considered to be contaminated with values increasing as the duration of the surgery extends (Beldi et al., 2009; Dellinger, 2011). No significance was highlighted between the duration of the surgery and the wound contamination, which appeared to be greater in surgery under 90 min (57% vs. 51%). Also for anesthesia, there was a higher frequency of contamination in procedures under 120 min (62.5% vs 50%) (Beal et al., 2000).

Hypothermia causes peripheral vasoconstriction with reduction of subcutaneous oxygen tension. It also compromises the subject's immune response. It was demonstrated in a randomised trial that the rate of surgical site infection tripled if the temperature dropped by 1.9°C from 36.5 to 34.6 (Kurz et al., 1996).

Patients showed hypothermia during surgery in 14 cases (22%) with a minimum temperature of 33°C (average = 34.6°C) although no statistically significant correlation with wound contamination was detected.

In literature hard tissue surgeries are considered more contaminated, because of the necrotic tissue present, necessary manual skills and the longer surgery and anaesthesia times (Cruse & Foord, 1980; Inacio et al., 2014; Kopp et al., 2015).

In this study soft tissue surgeries (n = 32; 21 wounds in the dog, 11 in the cat) compared to hard tissue surgeries (n = 32; 27 in the dog, 5 in the cat) were, although without obvious statistics, more contaminated than surgeries involving hard tissue (62% vs 50%).

In 25 subjects, with a total of 29 surgical wounds, intraoperative complications occurred: hypotension developed in 9 cases and more or less intense bleeding was reported in 6 cases. There was a higher contamination in subjects who, during the procedure, showed one or more complications, even if a statistical correlation has not been demonstrated; among these, hypotension was most incriminated, having high percentages of bacterial contamination (15% in the swab, 46% in the needle aspiration and 70% in the skin biopsy).

CONCLUSIONS

The contamination of the surgical wound is the major factor involved in the development of surgical site infection, in clinical practice. This can be analyzed as a predictive value to assess the subsequent possibility of infection development.

It was shown that there is a significant reduction in bacterial contamination in those cases where adhesive surgical drapes have been used: from the results obtained, between the two types of incision films the adhesive drape with betadine significantly reduce intraoperative contamination of the surgical site.

Based on the isolated aetiological agents, it can be assumed that both endogenous and exogenous bacteria cause the contamination of the surgical wound. Contamination represents only one of the precursors of bacterial infection: in Veterinary Medicine attention must, therefore, be paid to wound management even in the postoperative period, considering that bacteria isolated from infectious outbreaks are not always present at the end of surgery.

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EFFECT OF GENOTYPE AND SOWING DATE ON YIELD AND YIELD COMPONENTS OF FACULTATIVE WHEAT IN TRANSYLVANIA PLAIN

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Abstract

Field experiments were conducted for two successive seasons during 2017/2018 and 2018/2019 at ARDS Turda to asset the performance of three different facultative wheat genotypes under different sowing dates. The experiment was laid out in split plot design with three replicates and comprised of five dates of sowing, noted I to V (I-III sown in autumn, IV - V sown in spring), in main plots and three facultative wheat genotypes (Taisa, Ciprian and Lennox) in subplots. Two years results revealed that in autumn sowing conditions, facultative wheat sown at the end of Oct. - beginning of Nov. performed better in number of productive tillers/plant, number of grains/spike, weight of grains/spike and grain yield. In the 1st season, Taisa had the highest grain yield (7.80 t ha^{-1}), but in the 2nd Ciprian performed better (7.63 t ha^{-1}). In spring sowing conditions, the facultative wheat needs to be sown as early as possible, especially Taisa (long growth cycle, later heading time). A delay in spring sowing tends to decrease number of tillers/plant, number of productive tillers/plant, weight of grains/spike and grain yield. Lennox performed better than Taisa and Ciprian in both seasons.

***Key words**: facultative wheat, sowing date, yield components.*

INTRODUCTION

Following Olesen et al. (2011) and Valizadeh's et al. (2014) research, it was concluded that the wheat cultivation period, in all climate change scenarios, will be reduced, compared to the current situation. Possible reasons are the increase of the temperature rate and the acceleration of the wheat growth stages. In 2016, Bing et al. have shown that a global increase in temperature of 1^{0}C would lead to an overall decrease in wheat production by 4.1-6.4%. So, the researchers must be prepared to provide the main source of food for mankind. For that, they must know the genetic materials and the proper sowing conditions for each genotype and “cultivation strategies have to be developed based on each site’s characteristics” (Eriksson & Magnusson, 2015).
The grain yield of wheat is “affected by environmental conditions” (Pereira Costa et al., 2013) and “can be regulated by sowing time” (Ozturk et al., 2006; Aslani & Mehrvar, 2012).
In the world cultivation of cereals we can distinguish typical spring wheat genotypes sown in spring, typical winter wheat genotypes sown in autumn, and transitional wheat forms (facultative genotypes), sown both in autumn and spring (Wyzińska and Grabiński, 2018). Facultative wheat can be sown both autumn and spring and its requirements for vernalization (Muterko & Salina, 2018) are satisfied in 5-30 days to 5-10°C (Ceapoiu et al., 1984).
Optimum sowing dates provide favorable temperature to obtained maximum yield (Muhammad et al., 2015). In autumn, at tillering stage, optimum sowing date could produce good crop growth that increases the cold tolerance (Safdar et al., 2013). Fall - developed tillers contribute more to grain yield than do tillers developed in the late spring (Ozturk et al., 2006).
Late sowing wheat has negative consequences such as a poorer field emergence (Spink et al., 2000). In this case, the winter wheat plants

have a low resistance to winter frost, resulting loss density. Sowing into colder soils delay wheat emergence, so that the seed treatment with fungicide is really necessary. Late sowing of wheat causes reduction in number of tillers/m^2 (Muhammad et al., 2015), in plant height (Tahir et al., 2009), in days to heading and maturity (Sial et al., 2005), in number of kernel per spike (Refay, 2011), of thousand kernel weight (Wajid et al., 2004; Muhammad et al., 2015) and consequently causes lower grain yield (Sial et al., 2005; Tahir et al., 2009; Yajam & Madani, 2013; Sohail et al., 2014; Tadeusz, 2014; Muhammad et al., 2015). In spring, late sowing wheat causes a low density, so it is more exposed to the grassy weed infestations and the vegetation is prolonged in summer (Ion, 2010).

Early sowing wheat increase the risk of excessive growth in autumn and prolongs the duration of tillering (Aslani & Mehrvar, 2012). Early planting produce greater number of spikes/square meter, heavier grains and highest grain yield (Wajid et al., 2006). It can also leads to an increased incidence of autumn pest infestation (as flies), diseases transmitted by certain vectors such as wheat curl mites (wheat streak mosaic) and aphids (barley yellow dwarf). Another problem that can arise from sowing too early is the control of grassy weed infestations.

The grain yield of spring wheat is reduced in comparison to the winter forms because of a shorter growing season and less resistance to spring drought. But it has a very important asset "its higher grain quality then of winter wheat" (Koppel, 2008; Wyzińska & Grabiński, 2018). In 1995, Morison and Long showed that winter wheat is more vulnerable to climate changes due to its higher sensitivity to temperatures for proper flowering time and successful grain reproduction. So, higher temperatures in a winter season will lead to insufficient or failed vernalization and therefore to a lower grain yield of winter wheat (Yan et al., 2015).

Sown in autumn, facultative wheat suffers like spring wheat because of low winter temperatures, but less than the typical spring wheat, giving superior outputs to spring wheat.

Sown in spring, facultative wheat turned out to be more late and sensitive to drought and high temperatures (Ceapoiu et al., 1984).

In Transylvania Plain, the optimum sowing date of wheat in autumn is between 25 of September and 10 of October and in spring the second half of March (Muntean et al., 2014). So, the present study was conducted to evaluate the genetic yield potential of three facultative wheat genotypes under different sowing dates (in autumn and spring).

MATERIALS AND METHODS

Materials and methods

Field experiments were carried out during 2017/2018 (1st) and 2018/2019 (2nd) seasons at ARDS Turda on latitude 46° 35′ N, longitude 23° 47′ E and altitude of 345-493 m above Adriatic Sea level (Grecu et al., 2007), in Transylvania Plain, Romania. The experiment was established on a typical clay Chernozem soil, typical for the forest steppe encountered over half of the Transylvanian Plain. The agrochemical indexes for this soil type, in the arable layer, were determined by OSPA Cluj (2014). The average values of these indexes are: the soil reaction is neutral (pH = 6.81-6.84), humus content = 3.36-3.73%, total nitrogen content = 0.177-0.205%, potassium content = 220 -320 ppm in Amp, mobile phosphorus = 11-35 ppm in Amp.

The experiment was laid out in split plot design with three replicates. Sowing date (E) was the main plot factor and the genotype (S) was the subplot factor (Taisa, Ciprian and Lennox). In the two experimental years, the climatic conditions did not allow the sowing of experiences to take place on the same date (Table 1).

Table 1. Sowing date

Sowing date / Season	Autumn			Spring	
	I - control	II	III	IV - control	V
2017-2018	10.10.'17	02.11.'17	06.12.'17	15.03.'18	04.04.'18
2018-2019	10.10.'18	30.10.'18	16.11.'18	04.03.'19	18.03.'19

If in the 1st season we respected the optimal sowing period of wheat in spring, in the 2nd season we decided to rush it because of the high temperatures and the lack of precipitations from 2018 during April and May (Table 2), which affected the emerged and tillering of facultative wheat sown in April 4 (especially Taisa genotype).

Nitrogen fertilization was applied in two phases: N_{36} kg ha^{-1} in autumn and N_{105} kg ha^{-1} before heading.

All other agronomic practices (e. g. method of planting, seeding rate, planting depth, weeding and harvesting) were kept constant for all treatments. The surface of the harvestable plot had 7.5 m^2 and the seeding rate was 550 germinable seeds/square meter. Plots were harvested individually by Wintersteiger Plot Combine and grain yield was reported at uniform moisture content (14%).

The observations were recorded for plant height, flag leaf area, number of tillers per plant, productive tillers per plant, days to physiological maturity (to determinate the length of growing season), number of grains per spike, weight of grains per spike, thousand grains weight (TKW) and grain yield.

To determinate flag leaf area, the measurements were made according to the classical method, at the flowering stage, on samples of 30 wheat plants from each plot that were marked. For that, we used the formula:

A = b × leaf length × max leaf width (Montgomery, 1911, cited by Chanda and Singh in 2002), where b is a coefficient (b = 0.75).

The number of tillers per plant was determined at the end of tillering stage for each genotype by using a sample of 30 plants.

Productive tillers/plant was obtained by dividing number of spike m^{-2} to number of emerged plants m^{-2}.

Before harvesting, the marked wheat plants were taken to the laboratory in order to determine the number of grains per spike, weight of grains per spike and thousand grains weight (TKW).

Using Wintersteiger microbatosis, each spike was beaten individually. The grains resulted from each spike were counted manually, then weighed with a high precision balance. The thousand grains weight (TKW) was determined by dividing the grains weight to the numbers of grains and then multiplying by 1000.

The grain yield obtained from each plot (kg/7.5 m^2) was weighed with a high precision balance, reported at uniform moisture content (14%) and then estimated in t ha^{-1}.

Climatic conditions

Climatic data during the investigated period (2017-2019) indicate that the years when the researches were conducted were different, both in rainfall quantities and level of temperatures (Table 2), as compared with a long term average (1957-2017).

Statistical analysis

The collected data were analysed statistically by the standard analysis of variance (ANOVA), using the Polifact program.

Table 2. Climatic conditions of the experimental area

Month	2017 - 2018				2018 – 2019				60 years average	
	Rainfall (mm)		Temperatures (°C)		Rainfall (mm)		Temperatures (°C)		Rainfall (mm)	Tempera-tures (°C)
	m.a.*	dev.**	m.a.*	dev.**	m.a.*	dev.**	m.a.*	dev.**		
August	36.1	-20.5	22.3	3.0	38.2	-18.4	22.3	3	56.5	19.3
Sept.	56.2	13.7	15.8	0.7	29.8	-12.7	16.7	1.6	42.5	15.1
Oct.	49.2	13.6	11.6	2.1	26.8	-8.8	12.7	3.2	35.6	9.5
Nov.	30.8	2.3	4.9	1.0	29.6	1.1	6.0	2.1	28.5	3.9
Dec.	20.7	-6.4	1.0	2.4	58.3	31.2	-0.9	0.5	27.1	-1.4
Jan.	16.7	-5.1	0.2	3.6	46.0	24.2	-2.2	1.2	21.8	-3.4
Feb.	33.4	14.6	-0.3	0.6	14.7	-4.1	1.7	2.6	18.8	-0.9
March	40.9	17.3	3.3	-1.4	12.3	-11.3	7.3	2.6	23.6	4.7
April	26.2	-19.7	15.3	5.4	62.6	16.7	11.3	1.4	45.9	9.9
May	56.8	-11.9	18.7	3.7	152.4	83.7	13.6	-1.4	68.7	15.0
June	98.3	13.5	19.4	1.5	68.8	-16.0	21.8	3.9	84.8	17.9
July	85.7	8.6	20.4	0.7	35.0	-42.1	20.4	0.7	77.1	19.7
August	38.2	-18.4	22.3	3.0	63.8	7.2	22.1	2.8	56.5	19.3

*monthly average; **deviation;

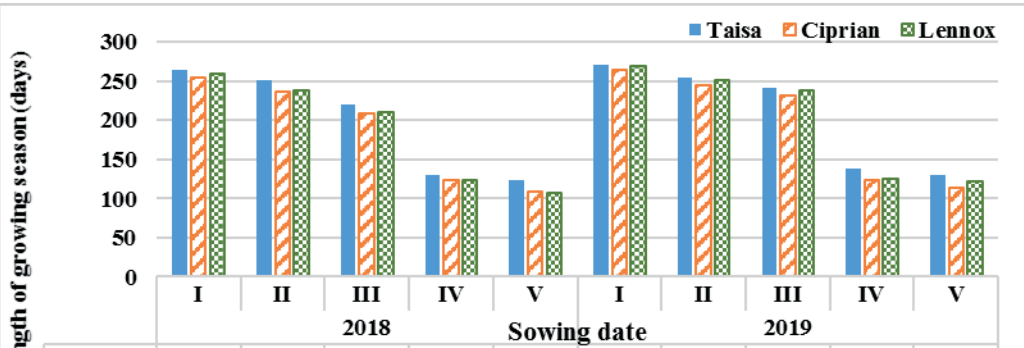
RESULTS AND DISCUSSIONS

Length of growing season

In order to establish the precocity of studied facultative wheat genotypes, we determined the length of growing season which is presented in Figure 1. It is obviously that, it decreases when the sowing date is delayed, regardless of the sowing time (autumn or spring).

If in the spring of 2018, respectively in April and May, we have experienced an acute lack of precipitations (deviation of -19.7 and -11.9 mm compared to the m.a.) and high temperatures (deviations of +5.4 and 3.7°C compared to the m.a.) which determined the acceleration of growth and development stages of wheat, the physiological maturity was reached in a shorter time, in 2019, in the same months, we were confronted with an excess of precipitation (deviation of +16.7 and +83.7 mm compared to the m.a.) and slightly lower temperatures (deviations of +1.4 and -1.4°C compared to the m.a.), so the growing season was longer. A shorter number in days to maturity caused by high temperature was also reported by Nahar et al. in 2010.

Analysing Figure 1, it can be recorded that Taisa is a facultative wheat genotype with a longer growth cycle, being followed by Lennox and Ciprian. Depending on the climatic conditions and sowing date, Taisa can reach a length of growing season of 220-271 days sown in autumn and 123-138 days sown in spring. Lennox can reach a length of growing season of 211-270 days sown in autumn and 107-126 days sown in spring. Ciprian can reach a length of growing season of 208-264 days sown in autumn and 109-123 days sown in spring.



	I	II	III	IV	V	I	II	III	IV	V
Taisa	265	251	220	130	123	271	255	241	138	130
Ciprian	255	236	208	123	109	264	244	231	123	114
Lennox	259	239	211	123	107	270	251	238	126	122

Figure 1. Length of growing season

Plant height

In both seasons, in autumn/spring sowing conditions, the plant height of facultative wheat was significantly influence by genotype (Table 3). Sown in autumn, the wheat plants were higher than sown in spring (Table 4). Lennox was the highest genotype, being followed by Ciprian and Taisa, in both seasons.

In the 1st season, sowing date had a very significant influence on plant height when facultative wheat was sown in autumn and a significant one, when it was sown in spring. In the 2nd season, at both sowing conditions, sowing date and the double interaction (S x E) had no influence on plant height (Table 3). So, the observed differences could be referred to environmental effect. In autumn sowing conditions, the highest difference (14.56 cm) was recorded in the 1st season (Table 5). A decrease of plant height in late autumn sowing can be attribute to a shorter growing period. The earliest sowing date may have enjoyed the better environmental conditions which resulted into the tallest plants. Similar results were reported by Sial et al. (2005). Also, Muhammad et al. (2015) reported that "late sowing of wheat caused reduction" in plant height.

Sown in mid-March, the plant height increased in the 2nd season with 17.78 cm because of the rains that fell in April-May (Table 5).

Flag leaf area

It is considered that the flag leaf has an essential role in the total productivity of the wheat plant, being the last to dry. The flag leaf can intercept a considerable amount of light energy which it converts into carbohydrates which will be translocated to the grain (Dobre & Lazăr, 2014).

The analysis of variance indicates significant differences for flag leaf area among the facultative wheat genotypes in both seasons (Table 3). For all genotypes, the highest values for flag leaf area were recorded in the 2nd season (Table 4). Sown in autumn, Taisa had the highest flag leaf area (30.10 cm^2) being followed by Lennox (26.08 cm^2) and Ciprian (25.10 cm^2). Sown in spring, Ciprian (34.13 cm^2) and Taisa (33.63 cm^2) had the highest values of flag leaf area and Lennox had the lowest (24.49 cm^2). In both seasons, sown in spring, the values of flag leaf area are slightly increased in Taisa and about the same in Lennox. Ciprian genotype had a different behaviour due to its short growth cycle and climatic conditions. So, in the 1st season, sown in spring, Ciprian genotype had the value of flag leaf area lower with 5.28 cm^2 than sown in autumn. In the 2nd season, sown in spring, the same genotype had the value of flag leaf area higher with 9.03 cm^2 than sown in autumn.

Sowing date (E) had a very significant influence on flag leaf area only in the 2nd season, in autumn sowing conditions. It had a non-significant effect on flag leaf area for all the other sowing conditions. On the other hand, the double interaction (S x E) has strongly influenced the flag leaf area in the 2nd season (Table 3).

In the 1st season, in autumn sowing conditions, as the sowing date is delayed, the flag leaf area decreases, but in spring sowing conditions, it increases (Table 5). In the 2nd season, the situation was exactly the opposite: in autumn sowing conditions, as the sowing date was delayed, the flag leaf area increased and in spring sowing conditions, it decreased. Sown in mid-March, the flag leaf area of facultative wheat genotypes increased in the 2nd season with 12.38 cm^2 (Table 5). The results obtained in the 1st season (autumn sowing conditions) may be due to the decrease in temperatures.

Number of tillers/plant

In both seasons, in autumn sowing conditions, number of tillers per plant was highly influenced by the sowing date. The influence of genotype and double interaction (S x E) on number of tillers/plant was significant only for the 2nd season, in autumn sowing conditions. In spring sowing conditions, the genotype and the sowing date had a significant effect on number of tillers/plant for the 2nd season, being non-significant for the 1st, and the interaction between the experimental factors had a significant effect on it just for the 1st season (Table 3).

The tillering ability of the facultative wheat genotypes was expressed in the 2nd season, at both sowing conditions (Table 4). Taisa genotype is the most stable for this character because, sown in autumn or spring, it produces the same number of tillers/plant. Lennox genotype had a different behaviour: in the 1st season, sown in autumn, it produced a higher number of tillers/plant than sown in spring; in the 2nd season was exactly the opposite (because in autumn it was affected by wheat dwarf virus and flies, which were favorited by the high temperatures and lack of precipitations from Oct. and Nov.).

Sown in autumn, the highest number of tillers/plant was obtained in both seasons at sowing date I (10 Oct.). So, a delayed sowing in autumn leads to a smaller number of tillers/plant (Table 5). A less number of tillers/plant in late sowing can be attribute to the fact that the temperature wasn't according to the tillering requirement. Differences in number of tillers/plant among genotypes might be attribute to their genetic diversity. These results are in accordance with those of Tahir et al. (2009).

In spring sowing conditions, the highest number of tillers/plant was obtained in the 2nd season, when the sowing took place at the beginning of March (4.03 tillers/plant). Compared to the 1st season, in the 2nd one, the tillering and the growth of tillers was positively influenced by the rainfall that occurred in April and May. Also, a late sowing of facultative wheat in spring causes a reduction in number of tillers/plant, it decreasing from 4.03 to 3.40 tillers/plant.

Productive tillers/plant

In autumn sowing conditions, the genotype, the sowing date and the interaction between them had a very significant influence on productive tillers/plant. In spring sowing conditions, if the genotype had a very significant influence on this parameter, the sowing date and the S x E interaction had just a significant influence on it (Table 3).

In Table 4, the comparison between the genotypes indicates that sown in autumn, in both seasons, Ciprian genotype had the highest number of productive tillers/plant. Sown in spring, in the 1st season, Ciprian genotype maintains its position, but in the 2nd season, Lennox genotype leads with 2.43 productive tillers/plant. So, the studied genotypes have different capacity of tillering and therefore the obtained grain yield depends on ability of tillers/plant to performed, regardless the climatic conditions.

Analysing Table 5, we can observe that in both seasons, in autumn sowing conditions, the number of productive tillers/plant is higher than in spring sowing time. It is in conformity with Ozturk et al. (2006) who reported that "fall-developed tillers also contribute more to grain yield than do tillers developed in the late spring". Sown in autumn, the best sowing date to obtain the highest number of productive tillers/plant was II (to the end of Oct. - beginning of Nov.), which is reflected in the obtained grain yields. Sown in spring, in both seasons, the best sowing date to obtain the highest number of productive tillers/plant was IV (from the beginning to the middle of March).

Number of grains/spike

Number of grains/spike was significantly influenced by genotype in both seasons and in both sowing conditions (Table 3). In the 1st season, if in autumn sowing conditions, the sowing date and the double interaction had a significant influence on number of grains/spike, in spring sowing conditions these factors had no statistical influence on it. Also, in the 2nd season (both sowing conditions), it was very affected by sowing date and S x E interaction (Table 3).

Analysing Table 4, we concluded that, in the 1st season (both sowing conditions), Taisa genotype produced the highest number of grains/spike (47.07 - sown in autumn, 47.35 - sown in spring), being followed by Lennox genotype (43.19 - sown in autumn, 38.13 - sown in spring) and Ciprian genotype (32.60 - sown in autumn, 37.38 - sown in spring). In the 2nd season, in spring sowing conditions, the order of the genotypes according to the mean values of number of grains/spike was the same as in the 1st season, but in autumn sowing conditions, the order of the genotypes was: Lennox (47.90), Taisa (39.87) and Ciprian (39.00).

In Table 5 is presented the influence of sowing date on number of grains/spike. In autumn (both seasons), late sowing caused an increase of number of grains/spike, which is not in accordance with the results obtained by Refay (2011) and Muhammad et al. (2015). It ranged from 35.51 to 46.40 in the 1st season and from 41.57 to 43.10 in the 2nd season. This fact can be attributed to a lower tillering. Sown in spring, the highest number of grains/spike was obtained in the 1st season. In both seasons, the best values of this parameter were obtained when the facultative wheat was sown in mid-March.

Weight of grains/spike

In autumn sowing conditions, in both seasons, genotype and S x E interaction had a significantly influence on weight of grains/spike, while sowing date had low or non-significant influence on it (Table 3). In spring sowing conditions, in the 2nd season, genotype, sowing date and the interaction between them had no influence on weight of grains/spike. In the 1st season, it wasn't influence by genotype and sowing date, but the double interaction recorded a significant one ($p > 5\%$).

Sown in autumn, in the 1st season, Taisa recorded the highest weight of grains/spike (1.88 g), being followed by Lennox (1.77 g) and Ciprian (1.43 g). In the 2nd season, the order of the studied genotypes was another: Lennox (1.86 g), Ciprian (1.66 g) and Taisa (1.43 g). All these results are presented in Table 4. Also, sown in spring, in the 1st season, Taisa had the highest weight of grains/spike (1.63 g) and Ciprian the lowest (1.48 g), but in the 2nd season, Taisa was still the leading one with 1.30 g, while Lennox was the last one with 1.12 g. Obviously, sown in autumn, the facultative wheat plants recorded higher mean values of this parameter than sown in spring.

Analysing Table 5, we can see that a late autumn sowing date leads to an increase of weight of grains/spike with 0.42 g (1st season). In the 2nd season, same conditions, the weight of grains/spike didn't change with the seeding delay (1.64 g). Sown in spring, the facultative wheat genotypes recorded the highest values of this parameter in the 1st season (1.62 g in sowing date IV and 1.47 g in sowing date V).

Thousand kernel weight (TKW)

In the 1st season, in autumn sowing conditions, genotype and sowing date had a significant effect on TKW, while their interaction had no influence on it. On the other hand, in the 2nd season, the experimental factors and their interaction had a very significant influence on this parameter. In spring sowing conditions, TKW was highly influenced by genotype and S x E interaction (in both seasons); sowing date had significantly influenced TKW only in the 2nd season, being non-significant in the 1st (Table 3).

In both seasons, sown in autumn, Ciprian genotype recorded the highest mean values of TKW, being followed by Lennox and Taisa (Table 4). Comparing the results obtained in the 1st season with those obtained in the 2nd (autumn sowing conditions), it can be noted that TKW of the studied genotypes was higher in the 1st season with 4.33 g for Taisa, 1.4 g for Ciprian and 2.09 g for Lennox.

Sown in spring, in the 1st season, Lennox had the best value of TKW (39.90 g), being followed by Ciprian (39.59 g) and Taisa (34.50 g). Sown in spring, in the 2nd season, Ciprian had the higher TKW (34.48 g), being followed by Taisa (30.41 g) and Lennox (28.47 g). The major loss from one season to another was recorded by Lennox (11.43 g). Also, in Table 4, it can be noted that TKW of the studied genotypes was higher in autumn sowing conditions than in spring ones, in both seasons. It can be attributed to a longer growth period.

The fact that Taisa genotype has the lowest TKW is due to its long growth cycle and later heading time, so the filling of grains is affected by drought.

The results from Table 5 shows that, in autumn sowing conditions, the lowest TKW was recorded in sowing date II in the 1st season (40.51 g) and in sowing date III in the 2nd season (38.08 g). In spring sowing conditions, the highest TKW was recorded at mid-March sowing time (39.33 g - 1st season, 31.92 g - 2nd season).

Grain yield

Genotype, sowing date and the interaction between them had highly influenced the grain yield of facultative wheat in both seasons, in autumn sowing conditions ($p > 1\%$). In spring sowing conditions, in the 2nd season, the influence of sowing date (E) and S x E interaction was not significant ($p < 5\%$) on the grain yield; only the genotype had a significant one ($p > 1\%$) on it. In the 1st season, grain yield was significantly influenced by genotype and S x E interaction ($p > 1\%$); also, sowing date had a significant effect on it ($p > 5\%$). All these results are presented in Table 3.

Analysing Table 4, the results shows that in the 1st season, in autumn sowing conditions, Taisa led with a grain yield of 7.27 t ha^{-1}, while in the 2nd season Ciprian stand out with a grain yield of 7.35 t ha^{-1}. In spring sowing conditions, in both seasons, Lennox has recorded the best grain yields (6.79 t ha^{-1} - 1st season, 5.67 t ha^{-1} - 2nd season), being followed by Ciprian (6.13 t ha^{-1} - 1st season, 5.20 t ha^{-1} - 2nd season) and Taisa (4.85 t ha^{-1} - 1st season, 4.20 t ha^{-1} - 2nd season).

The results presented in Table 6 shows that in both seasons, in autumn sowing conditions, the sowing date II promoted a higher expression of grain yield potential to all genotypes. This is observed very well from the graphical representation of the average yield over the two seasons (Figure 2) resulting from the E x S interaction. The lowest grain yield was recorded in the last sowing date. In spring sowing conditions, in both seasons, the first sowing date (IV) promoted a higher expression of grain yield potential to all genotypes (Table 6 and Figure 3).

The yield decrease of Taisa genotype can be attribute, in both seasons, in both sowing conditions, to a lower number of productive tillers/plant and to a poor filling of grains due the long growth cycle.

Table 3. Mean squares from analysis of variance for various characteristics obtained from three wheat cultivars sown under five sowing dates in 2017/2018 and 2018/2019 seasons at Turda

Source of variance	Genotype (S)		Sowing date (E)		Interaction (S x E)	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Sown in autumn						
Plant height (cm)	684.703***	375.111**	574.925***	88.111^{ns}	15.703*	18.055^{ns}
Flag leaf area (cm^2)	104.037**	63.151***	34.291^{ns}	88.134***	9.409^{ns}	36.048***
No. of tillers/plant	3.633^{ns}	10.033**	199.033***	80.033***	2.316^{ns}	6.666**
Productive tillers/plant	0.128***	0.092***	0.282***	0.160***	0.015***	0.059***
No. of grains/spike	504.669**	216.743***	266.778*	5.453***	121.613**	12.383**
Weight of grains/spike (g)	0.508**	0.403***	0.417*	0.001^{ns}	0.222**	0.014*
TKW (g)	34.517*	98.100***	11.325*	6.810***	4.876^{ns}	2.171**
Grain yield (t ha^{-1})	2.017**	11.643***	4.007***	2.632**	0.412**	2.724**
Sown in spring						
Plant height (cm)	289.041**	1165.167***	144.500*	2.722^{ns}	66.291**	7.722^{ns}
Flag leaf area (cm^2)	183.799**	176.719***	36.352^{ns}	1.602^{ns}	87.228*	17.952***
No. of tillers/plant	2.216^{ns}	8.016*	0.816^{ns}	6.016*	14.516*	5.416^{ns}
Productive tillers/plant	0.119***	0.242***	0.123*	0.242*	0.052**	0.015*
No. of grains/spike	184.843**	72.665***	0.500^{ns}	15.680***	1.545^{ns}	11.285**
Weight of grains/spike (g)	0.036^{ns}	0.158^{ns}	0.099^{ns}	0.228^{ns}	0.152*	0.147^{ns}
TKW (g)	55.110**	56.528***	32.026^{ns}	11.472***	81.416**	6.470***
Grain yield (t ha^{-1})	5.847**	3.351**	6.303*	1.797^{ns}	3.615**	0.119^{ns}

*, **, *** - significant at the 5%, 1% and 0.1% probability levels, respectively; ^{ns} - non significant

Table 4. Mean values of yield components and yield of three facultative wheat cultivars in 2017/2018 and 2018/2019 seasons at ARDS Turda

Source of variation	Taisa		Ciprian		Lennox		LSD 5%	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Sown in autumn								
Plant height (cm)	86.78^{000}	91.00^{00}	88.22^{000}	98.11^{ns}	102.56***	103.89**	2.02	3.96
Flag leaf area (cm^2)	21.76^{ns}	30.10***	22.33^{ns}	25.10^{000}	16.17^{00}	26.08^{000}	2.31	0.50
No. of tillers/plant	2.97^{ns}	3.63^{ns}	3.03^{ns}	3.27^{ns}	3.60^{ns}	2.50^{0}	0.70	0.59
Productive tillers/plant	2.16^{000}	2.28^{ns}	2.40***	2.35***	2.28^{ns}	2.15^{000}	0.01	0.02
No. of grains/spike	47.07*	39.87^{000}	32.60^{00}	39.00^{000}	43.19^{ns}	47.90***	4.87	0.75
Weight of grains/spike (g)	1.88*	1.43^{000}	1.43^{00}	1.66^{ns}	1.77^{ns}	1.86***	0.14	0.06
TKW (g)	40.27^{ns}	35.94^{000}	43.92^{ns}	42.52***	40.88^{ns}	38.79^{00}	2.54	0.17
Grain yield (t ha^{-1})	7.27**	5.48^{000}	6.39^{000}	7.35***	7.12^{ns}	5.29^{000}	0.20	0.27
Sown in spring								
Plant height (cm)	73.58^{0}	79.00^{000}	72.75^{0}	91.67^{ns}	85.17***	106.83***	3.16	3.60
Flag leaf area (cm^2)	25.83*	33.63***	17.05^{ns}	34.13***	15.59^{ns}	24.49***	4.72	0.38
No. of tillers/plant	3.00^{ns}	3.60^{ns}	2.45^{ns}	3.15^{ns}	2.40^{ns}	4.40^{ns}	0.79	0.89
Productive tillers/plant	1.92^{000}	2.04^{000}	2.20***	2.15^{ns}	2.08^{ns}	2.43***	0.05	0.08
No. of grains/spike	47.35*	42.50***	37.38^{ns}	35.55^{000}	38.13^{ns}	39.35^{ns}	5.34	1.03
Weight of grains/spike (g)	1.63^{ns}	1.30^{ns}	1.48^{ns}	1.23^{ns}	1.52^{ns}	1.12^{ns}	0.19	0.19
TKW (g)	34.50^{000}	30.41^{000}	39.59*	34.48***	39.90*	28.47^{000}	1.46	0.13
Grain yield (t ha^{-1})	4.85^{000}	4.20^{000}	6.13^{ns}	5.20^{ns}	6.79**	5.67**	0.45	0.35

Mean values of genotypes were considered control;
*, **, *** - significant at the 5%, 1% and 0.1% probability levels, respectively; ^{ns} – non significant

The highest yield loss of Taisa (2.95 t ha^{-1}) was recorded in spring sowing conditions, 1st season, so it is restricted in spring to be sown in the first half of March.

Analysing the grain yield obtained by Lennox genotype, in the 2nd season, in autumn sowing conditions, we can notice that the lowest value (3.73 t ha^{-1}) was at the sowing date I. This high decrease can be explained by fewer productive tillers/plant and lower TKW. These were caused by barley yellow dwarf disease transmitted by certain vectors such as wheat curl mites and aphids whose flight was favored by climatic conditions (high temperatures and lack of precipitations from Oct. and Nov. - see Table 2). So, Lennox being a foreign genotype, less adapted to our conditions, in the warm autumn it needs to carry out appropriate phytosanitary treatments.

Table 5. Mean values of yield components and yield at different sowing dates in 2017/2018 and 2018/2019 seasons at ARDS Turda

Source of variation		Plant height (cm)	Flag leaf area (cm^2)	No. of tillers/ plant	Productive tillers/plant	No. of grains/ spike	Weight of grains/spike (g)	TKW (g)	Grain yield (t ha^{-1})
Sowing date		Sown in autumn							
I Ct*	1st season	97.89	21.61	6.03	2.38	35.51	1.51	42.74	7.37
	2nd season	100.33	24.38	4.97	2.11	41.57	1.64	39.58	5.72
II	1st season	96.33^{ns}	20.76^{ns}	2.57^{000}	2.38^{ns}	40.94^{ns}	1.64^{ns}	40.51^{00}	7.26^{ns}
	2nd season	98.44^{ns}	26.38***	2.60^{000}	2.30***	42.10*	1.66^{ns}	39.59^{ns}	6.66**
III	1st season	83.33^{000}	17.89^{ns}	1.00^{000}	2.07^{000}	46.40*	1.93*	41.83^{ns}	6.16^{000}
	2nd season	94.22^{ns}	30.52***	1.83^{000}	2.37***	43.10***	1.64^{ns}	38.08^{000}	5.73^{ns}
LSD 5%	1st season	1.71	4.62	0.61	0.04	7.31	0.28	1.28	0.24
	2nd season	7.53	0.21	0.72	0.05	0.45	0.15	0.28	0.45
		Sown in spring							
IV Ct*	1st season	74.33	18.07	2.50	2.15	41.12	1.62	39.33	6.51
	2nd season	92.89	31.04	4.03	2.32	38.20	1.15	30.32	5.34
V	1st season	80.00*	20.91^{ns}	2.73^{ns}	1.99^{0}	40.79^{ns}	1.47^{ns}	36.66^{ns}	5.33^{0}
	2nd season	92.11^{ns}	30.45^{ns}	3.40^{0}	2.09^{0}	40.07***	1.28^{ns}	31.92***	4.71^{ns}
LSD 5%	1st season	2.90	5.63	0.71	0.09	9.02	0.32	2.82	0.58
	2nd season	1.26	0.63	0.58	0.10	0.23	0.13	0.18	0.66

*Ct – control; *, **, *** - significant at the 5%, 1% and 0.1% probability levels, respectively; ns – non significant;

Table 6. Interaction between sowing date and genotype on grain yield t ha^{-1} in 2017/2018 and 2018/2019 at ARDS Turda

Sowing date	Genotype	1st season	2nd season	Average
	Sown in autumn			
I Ct*	Taisa	7.76	5.87	6.815
	Ciprian	6.44	7.55	6.995
	Lennox	7.90	3.73	5.815
II	Taisa	7.80^{ns}	5.98^{ns}	**6.890**
	Ciprian	6.77^{ns}	7.63^{ns}	**7.200**
	Lennox	7.20^{00}	6.37***	**6.785**
III	Taisa	6.26^{000}	4.58^{00}	5.420
	Ciprian	5.95^{0}	6.86^{0}	6.405
	Lennox	6.27^{000}	5.76***	6.015
	LSD 5%	0.37	0.59	
	Sown in spring			
IV Ct*	Taisa	6.32	4.68	5.500
	Ciprian	6.15	5.42	5.785
	Lennox	7.07	5.92	6.495
V	Taisa	3.37^{00}	3.73^{0}	3.550
	Ciprian	6.11^{ns}	4.97^{ns}	5.540
	Lennox	6.51^{ns}	5.42^{ns}	5.965
	LSD 5%	0.74	0.74	

*Ct – control; *, **, *** - significant at the 5%, 1% and 0.1% probability levels, respectively; ns – non significant

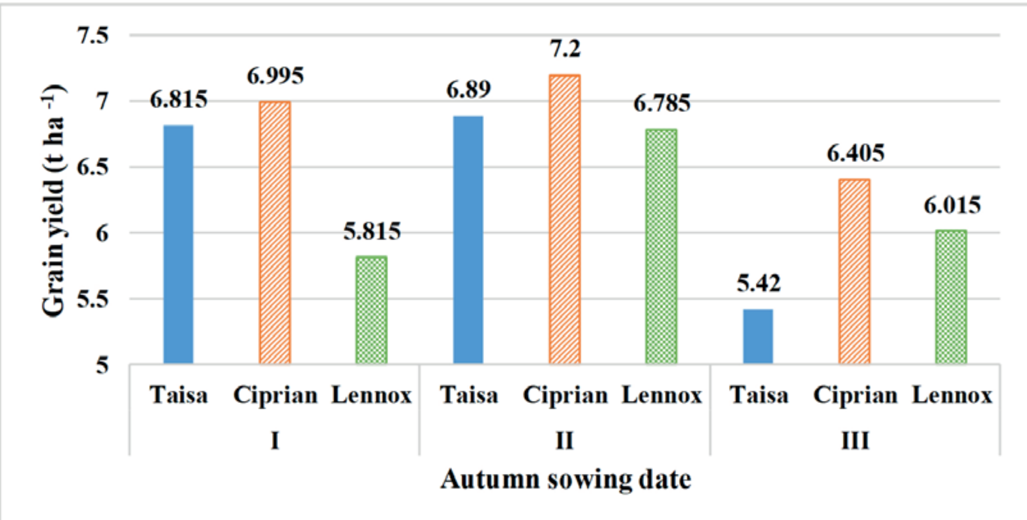


Figure. 2 Average yield in autumn sowing conditions

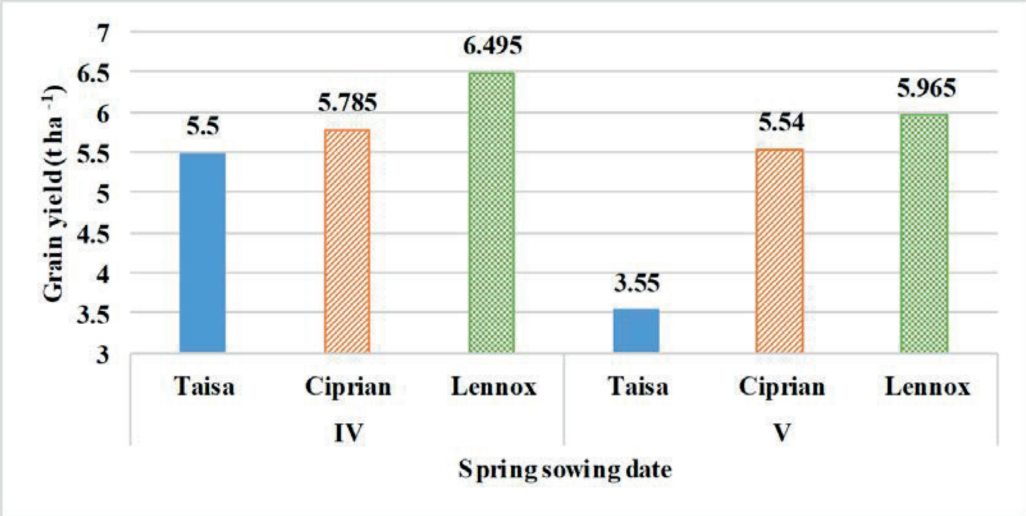


Figure. 3 Average yield in spring sowing conditions

CONCLUSIONS

In autumn sowing conditions, to get the best grain yields, the facultative wheat genotypes are suitable to be sown at the end of October – beginning of November. A delay in sowing tends to decrease the plant height, number of tillers/plant, number of productive tillers/plant, TKW and grain yield. On the other hand, the number of grains/spike and the weight of grains/spike tend to increase.

In spring, to get an appropriate grain yield, the facultative wheat needs to be sown as early as possible, especially Taisa (long growth cycle, later heading time). This genotype is restricted in spring to be sown until 15 of March. A delay in sowing tends to decrease number of tillers/plant, number of productive tillers/plant, weight of grains/spike and grain yield.

In unappropriated climatic conditions, each genotype response on its owns ways to achieve the best grain yield. So, Ciprian had a higher tillering and productive tilers/plant and obtained the highest TKW because of its short growth cycle, early heading time and Taisa and Lennox had a lower productive tillers/plant, but a higher number of grains/spike.

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WATER USE EFFICIENCY IN GREENHOUSE SYSTEMS AND ITS APPLICATION IN HORTICULTURE

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Abstract

Traditional culture system (TCS) in open field is high demanding in water and other natural resources and thus has driven the development of protected cultivation systems with innovative horticultural growing techniques. Greenhouse systems and soilless culture system (SCS) can allow obtaining high yields and improving water use efficiency especially in marginal and arid regions. Desert areas have tapped the opportunity arising from technical innovation to create new chances for horticulture development with important social and environmental impacts; today these advanced techniques can be successfully adopted in every context in which a high-water use efficiency is needed. Indoor and vertical farming represent new possible cultivation strategies for urban and peri-urban areas where the SCS plays an important role. Integrated systems must be adopted for lowering the production costs such as the use of innovative lighting systems and ICT controlling units for real time management. In fact, the application of highly specialized growing techniques represents an efficient tool to increase sustainable agricultural productions, thanks to the interaction between growers, supply chain partners, research institutes and governmental agencies.

Key words*: case studies, hydroponics, innovative production systems, soilless culture systems, WUE.*

INTRODUCTION

The industrialization of agriculture led to increase homogeneity across food systems as farming techniques and markets become more standardized (Lyson, 2004; Lyson and Guptill, 2004). Therefore, the complex social relationships underlying agriculture and ecosystem service provision have become less visible. The main objectives of the agricultural systems are competitiveness, efficiency and environmental protection through innovative practices and environmentally friendly technologies (Lundqvist et al., 2008; Nellemann et al., 2009; FAO, 2013). The request of high-quality horticultural products is increasing, due to the growing interest of consumers in fresh products of high organoleptic, nutritional, and functional value. The quality of fresh horticultural commodities has been described as "*a dynamic composite of their physicochemical properties and evolving consumer perception, which embraces organoleptic, nutritional and bioactive components*". The increase of input costs and the stabilisation of sale prices of produce have driven greater investments in technology, crop yield (which is increased more than two-fold from 1975) and resource efficiency, creating a dynamic system: the intensive horticulture (Galdeano-Gómez et al., 2013). The effect of new cultivars and more efficient production system management are detectible in terms of increased production and productivity in protected cultivation. The protected cultivation systems allow a reduction of the transpiration, respect to open field in which circa the 98% of the water taken up by an annual plant is lost (FAO, 2004) (Figure 1).
Water is fast becoming a scarce resource in many areas of the world (FAO, 2016) and its management is one of the most important political, social, and economic issues of the twenty-first century (Abou Hadid, 2013). The United Nations World Water Development Report (2018) stated that nearly 6 billion peoples will suffer from water scarcity by 2050 as a result of increasing demand for water

(Boretti and Rosa, 2019). Agriculture is the largest water user worldwide, accounting for 70% of total freshwater withdrawals on average, but these amounts can reach as much as 95% in some developing countries. Agriculture activity, if not well managed, can be a major source of water pollution from excessive nutrients supply, pesticides, and other contaminants, which if unmanaged can lead to significant social, economic, and environmental costs (FAO, 2016). Low irrigation efficiency can be primarily attributed to water mismanagement, technical distribution problems and poor maintenance of irrigation structures (Abou Hadid, 2013). The low water availability is forcing many growers to use water with relatively high salt concentration for crop irrigation or to improve methods, techniques, and management practices in agricultural and horticultural production. It is estimated that 18% of cropland is irrigated accounting for ca 40% of productivity with an irrigation efficiency of 40-65% for furrow irrigation, 45-75% for basin irrigation, 60-70% for sprinkler irrigation, 80% for localized irrigation (Abou Hadid, 2013). Furthermore, more strict environmental regulations related to water use are now prevailing in many countries.

Figure 1. Cultivation in areas with water scarcity

Awareness of the pollution, associated with intensive agriculture, forces greenhouse growers to adopt environment-friendly cultivation methods, such as closed soilless culture (Vox et al., 2010; Gruda, 2009) (Figure 2). Soilless culture system (SCS) is a cropping system that gained popularity worldwide in the 1930s in plant nutrition experiments (Engindeniz, 2004). The nutrient solution (NS) supplied to the growing system can be collected and reused (closed-loop system) or lost as drainage (open-loop system).

Figure 2. Soilless cultivation in open field and protected cultivation in areas with water scarcity

Closed SCSs are environmentally friendly, reducing the amount of the water and nutrients used for each growing cycle. However, the management costs are higher and require skilled agronomists for controlling the NS composition and properties during recirculation. Open SCSs are easier in the management, with a NS freshly prepared and delivered to the crops. The disadvantages of these systems are related to the higher amount of water and nutrients used. SCS consents to produce raw vegetables by using NS with or without solid inert material (e.g. rockwool, turf stone, clay granules, sawdust, flexible polyurethane foaming blocks, composed hardwood bark, peat) as support to the roots (Engindeniz, 2004). Inert substrates are also suitable to significantly reduce the evapotranspiration that is of particular interest in areas with adverse growing conditions (Gruda, 2009). Currently, about 3.5% of the worldwide area cultivated under tunnels and greenhouses for vegetables production adopts the soilless agriculture techniques based on hydroponic solution, such as floating systems, nutrient film technique or aeroponics, (Sambo et al., 2019). This significant diffusion at the field scale undoubtedly highlights the presence of many advantages such as plant protection prevention, product quality improvement, improving water use efficiency (WUE), and ensuring high yields (Kinoshita et al., 2016; Ferrante et al., 2003; Nicola & Fontana, 2007; Fontana & Nicola, 2009; Nicola et al., 2016).

In SCS, the NS composition has to be adapted to the quality of water, the crop species, the growth stage, and the climatic conditions. Only the use of modern information and computer technologies can permit to pursue these objectives. Suitable automation technologies are frequently based on real-time measurement of parameters connected either to the greenhouse microclimate (e.g., solar radiation, vapor pressure deficit, air temperature) or to the growing media (GM) water status (water tension or content). SCS is highly productive, allowing controlling and managing efficiently the input usage precision, increasing the yield-related WUE, reducing waste (e.g. of N supply). It is suitable for producing vegetables with both a short culture cycle and a high planting density, increasing earliness and allowing the extension of the growing seasons (Fontana & Nicola, 2008; Rodríguez-Hidalgo et al., 2010; Grewal et al., 2011; Scuderi et al., 2011). SCS is also able to recycle water and nutrients because the drainage can be recovered; the reuse does not in general restrict crop yields while reducing the water source pollution (Grewal et al., 2011). In the past, SCS was used in open systems, thus the surplus of supplied water and of nutrients were lost reducing the efficiency, increasing waste and resulting in contamination of groundwater, particularly with nitrates and phosphates (Incrocci et al., 2006; Savvas et al., 2007). SCS is useful and suitable to apply positive stress to plants. Indeed, application of eustress (positive stress), such as salinity or nutritional stress, can elicit physiological responses and molecular mechanisms that cause the accumulation of bioactive compounds such as non-structural carbohydrates and health-promoting phytochemicals such as lycopene, β-carotene, vitamin C, and the overall phenolic content of tomato fruits (Rouphael and Kyriacou, 2018). In addition, salinity eustress can decrease the accumulation of anti-nutrient compounds such as nitrate, thanks to the antagonism in absorption between chloride and nitrate (Rodriguez-Ortega et al., 2017; Rouphael and Kyriacou, 2018). In Europe, the legislation drafted to reduce the environmental impact of horticulture stemming from fertilization runoff, is forcing greenhouse growers to invest in closed systems (Savvas et al., 2007). In addition, SCS can improve raw material quality at harvest avoiding over-head irrigations and the contact between NS and edible parts reducing microbial contamination, eliminating soil and chemical residue spoilage and can solve problems caused by soil-borne diseases (Fontana & Nicola, 2009). FAO (2004) reported that 1 m^3 of water consumption affects yield according to the cropping system used, ranging from 3 kg of tomato production obtained in open field system, to 10-12 kg in protected cultivation system to 30 kg in SCS in protected cultivation system. The water consumption to produce 1 kg of commercial yield of vegetables varies considerably from TCS in open field to SCS in greenhouse (tomato 123 L vs 13 L; cucumber 205 L vs 10 L; lettuce 96 L vs 5 L, respectively) (FAO, 2004). Jovicich et al. (2007) reported that greenhouse cucumber crops in Florida (USA) required 33% less of the water amount, 28% less nitrogen and 23% less potassium per kg of fruit compared with a field grown cucumber crop. Among the different SCSs, floating systems (FS) are relatively cheap and easy-to-use sub-irrigation systems that can be implemented either with a continuous flotation (FL) or with an ebb-and-flow flotation (EF) (Fontana & Nicola, 2009). FL consists of trays floating continuously on a water-bed or NS and requires relatively little maintenance or labour cost, resulting in a more efficient use of water and greenhouse space (Fontana & Nicola, 2008). EF is scheduled with drying (ebb) periods with discontinuous flotation or aerating the NS to reduce plant hypoxia for those vegetables as rocket which may suffer stress due to the consumption of oxygen dissolved in the NS if grown in FL (Son et al., 2006; Nicola et al., 2007; Fontana & Nicola, 2009). The EF system may allow 85% reduction of water use, 50% of fertilizer use, 50 to 60% of pesticide use, elimination of groundwater contamination and rare occurrence of foliar and soil-borne diseases (Son et al., 2006; Fontana & Nicola, 2009). With the nutrient film technique (NFT), a continuous or intermittent flow of NS pumped from a tank flows over the roots in a tube or tray and then returns to the tank assuring adequate aeration of the roots

(Cooper, 1979). Recycled NS must be monitored and checked regularly to adjust nutrient strength, conductivity, pH, and control the supply because if the pump stops working, plant roots are prone to drying out (Cooper, 1979; Wolosin, 2008). Cropland currently occupies about 1.53 billion hectares or 12% of the Earth's ice-free land and the most suitable area for cropping have already been converted to cropland (Kummu et al., 2012). Expansion is therefore often into marginal areas where crops productions are particularly exposed to extreme conditions and stress reducing productivity and adaptation (West et al., 2010; Kummu et al., 2012). Thus, beside to be widely used in developed countries, greenhouses and SCSs have been introduced in marginal areas or in arid lands where favourable climate conditions but problematic soil properties, water scarcity or erratic rainfall distribution exist (Massa et al., 2011; Gruda, 2009). Some authors have shown the importance of the different environmental conditions in the design of controlled structures and strategies, for which knowledge of the characteristics of each region is essential (Mazuela et al., 2012).

CASE STUDIES

1. Arid Regions

1.1. The Almería phenomenon

The Almería region is characterized by an intensive horticultural cropping system. Almería underwent an unprecedented transformation in Spain's recent economic history, thanks to the agricultural development (Aznar-Sánchez et al., 2011; Garcia-Caparros et al., 2017). This represents an example of how large-scale greenhouse agriculture can affect, both positively and negatively, the landscape (Aznar-Sánchez et al., 2011; Giagnocavo, 2012).

Almería is located in the autonomous community of Andalucía in the South East of Spain (Figure 3). The area is characterized by an extremely arid climate, with average temperature of 17-20°C, due to the proximity of the mountains which provide a protective boundary against cold northerly winds and winter storms (Tout, 1990), little temperature variations between day and night, about 3000 hours of annual sunshine and about 200-250 mm of rain per year (Cantón et al., 2003; Downward & Taylor, 2007; Aznar-Sánchez & Galdeano-Gómez, 2011).

Figure 3. The area of the Almería region with the intensive protected cultivation development (source: maps.google.com)

Because of the location, climate and lack of water, Almería has not being rapidly urbanized (Cantliffe & Vansickle, 2012) and until the end of the 1960s social and economic indicators characterized Almería as an underdeveloped and in a stage of decline province (Aznar-Sánchez et al., 2011). Environmental factors have played an important role in the province change, which led to the adoption of a production system more efficient, especially in the use of water, and more sustainable (Galdeano-Gómez et al., 2008; 2013). In the 1950s, agricultural production was mainly based on table grapes cultivation (produced on wire trellis systems covered with plastic to induce earliness) and citrus (Cantliffe & Vansickle, 2012). With modern transportation and the accessibility to other varieties, the table grape industry suffered a decline becoming an unproductive source of cropping for farmers, encouraging them to look for new sources of income (Wolosin, 2008). In that period the sand-plot (*enarenado*) technique was introduced (Figure 4). It consists in the preparation of a mechanically levelled soil surface, covered with a layer of compacted clay, fermented manure and sand or coarse grit (Tout, 1990). The National Institute for Colonization promoted the use of the technology necessary for extracting the abundant underground water resources compensating low and erratic rainfall, provided infrastructure for water and electricity use and encouraged people to settle in the area offering technical and financial advice (Giagnocavo et al., 2010; Aznar-Sánchez & Galdeano-Gómez, 2011; Giagnocavo, 2012). Consequently, farmers from the close rural areas were attracted by the proposition of easy access to land, the possibility of obtaining their own property and the great profitability (Baldock et al., 2010; Aznar-Sánchez et al., 2011). The new farms were supported by the adoption of several technological innovations developed and rapidly incorporated in the fields, including windbreaks to protect the sand-plots system from high winds and to prevent sand loss (Aznar-Sánchez et al., 2011).

Figure 4. Typical crop in *enarenado* system in Almería area

During the 1970s and 1980s, several grape cultivations were converted to vegetable fields because of the greater profitability and to the introduction of greenhouse and new farming techniques (Cantliffe & Vansickle, 2012). These innovations completely transformed the unproductive lands into prosperous farms, providing effective protection against weather and environmental conditions contributing to increase yields and precocity, allowing the extension of the growing season, the quality and water conservation (Ferraro García & Aznar-Sánchez, 2008; Aznar-Sánchez & Galdeano-Gómez, 2011). In 1975, approximately a quarter of the irrigated area of Almería was covered by greenhouses (Wolosin, 2008). The general horticultural market expansion and the increasing demand for off-season produce were the main reasons for the continued investment and farm development. Furthermore, this expansion was supported by several initiatives which were introduced for family-run farms and cooperative bank, including private financing structures and credit facilities offered by marketing farms (Aznar-Sánchez et al., 2011; Giagnocavo, 2012; Galdeano-Gómez et al., 2013).

In the 1980s the attention to the efficient use of irrigation water is grown and in 1984 the Government of Andalucía reduced the expansion of irrigated greenhouses with legal restrictions due to the over-exploitation of the water resources available (Wolosin, 2008). In the following years, the Government of Andalucía funded the construction of deeper wells, water supply lines and new irrigation pipes in response to water shortages and farmers' demand (Wolosin, 2008). Drip irrigation was mandatory applied to reduce water use and evapotranspiration loss, while increasing the efficiency of its distribution on the plants (Tout, 1990; Cantliffe & Vansickle, 2012). More precisely, in recent years, the fertigation is mostly adopted as agro-technique, providing a very good opportunity to minimize water and nutrients losses simultaneously (Garcia-Caparros et al., 2017; Incrocci et al., 2017). As a consequence, to the limitations in the use of water resources, Almería experienced little to no greenhouse construction from 1985 to 1989 (Wolosin, 2008). The Almería's agriculture consolidation took place in the 1990s with constant improvement in production technique, technological innovation, and marketing for the competitiveness maintenance (Aznar-Sánchez et al., 2011). Today, almost 90% of the greenhouse area in Almería produces soilless vegetables, which are grown on an artificial soil made by clay, sand, gravel, manure and either perlite or rockwool as soilless media, and the production has increased optimizing costs (Cantliffe & Vansickle, 2012; Giagnocavo, 2012). The land area dedicated to horticultural greenhouse farming has increased dramatically from approximately 3,000 ha in 1975 to more than 30,000 ha at present (Aznar-Sánchez et al., 2011; Garcia-Caparros et al., 2017; Parra et al., 2019). Nowadays, in Almería there are 36,000-

40,000 ha of greenhouse vegetable crops representing more than half of the total area of greenhouses in Spain dedicated to vegetables and the most concentrated greenhouse area in Europe. Particularly, the major greenhouse area is concentrated in the South-West of the province, at Campo de Dalías, with a concentration of over 16,000 ha of greenhouses making it one of the most important areas of intensive farming worldwide (Aznar-Sánchez & Galdeano-Gómez, 2011; Aznar-Sánchez et al., 2011). In Almería, the volume greenhouse production increased from 600,000 metric tons to 2.7 million metric tons in the last 30 years, representing about 25% of the national total production, making Almería the top vegetable growing province in Spain (Wolosin, 2008; Cantliffe & Vansickle, 2012). The grown interest in Almería production from national and international produce companies has brought additional changes to the greenhouse system and the expansion of auxiliary industries related to construction, maintenance, as well as to the distribution, packaging, seeding, recycling of produce and research centres (Aznar-Sánchez & Galdeano-Gómez, 2011; Aznar-Sánchez et al., 2011; Cantliffe & Vansickle, 2012). Almería vegetable production includes 30 different crop species, representing 90% of the total agricultural production, that are generally grown as winter crops (tomato, sweet pepper, cucumber, green beans, eggplant and certain squashes) and summer crops (various muskmelons, watermelons and zucchini) with production peaks in December-January and in May-June (Cantliffe & Vansickle, 2012; Garcia-Caparros et al., 2017). Half of the sweet peppers and a quarter of cucumbers and tomatoes consumed in Europe come from Almería indicates the importance of an all year production (Wolosin, 2008). Despite the economic success, the Almería production system has several structural weaknesses. The fast and intensive horticulture development occurred in the initial stage did not include any kind of territorial planning and organization and generated some negative drawbacks, also because for long time the main objective was to increase the productivity and to maximize the short-term economic gains (Aznar-Sánchez & Galdeano-Gómez, 2011; Aznar-Sánchez et al., 2011) and no attention was paid to environmental issues. In this sense, water quality and management are the most important factors (Castro et al., 2019). Due to overexploitation, water shortage and price increment occurred, while the abundant use of pesticides and fertilizers contributed to contamination of water resources and led to the progressive salinization of the soils (Tout, 1990; Aznar-Sánchez et al., 2011; Cuevas et al., 2019). The solution to these problems was the use of reservoirs and recycled water, progressive adaptation of SCS and the building, at the end of 1990s of two desalination plants (the biggest in Europe) (Colino Sueiras & Martínez Paz, 2002; Galdeano-Gómez et al., 2008; Aznar-Sánchez et al., 2011). The desalination plants are able to supply water for domestic and irrigation use despite the high-energy demand, which is only partially covered by renewable energy resources (Downward & Taylor, 2007). The development of Almería's horticultural sector may be useful in helping other areas to adapt and improve their agricultural systems especially when small-scale farming predominates (Galdeano-Gómez et al., 2013).

1.2. Technical innovation in Tunisian horticultural greenhouses

Many North African countries are affected by the most severe water shortages and by the greatest challenges in terms of future water availability because of the semi-arid climate, limited and variable rainfall and strong water evaporation (Frija et al., 2009). Starting from the 1980s, these countries have developed irrigation infrastructures planning the control of water resources, in order to increase their supply stability given that agriculture accounts for around 80% of the total water consumption (Shiklomanov, 2000; Döll, 2009; Frija et al., 2009; Kummu et al., 2012).

Rainfall in Tunisia varies from less than 100 mm per year in the South to more than 1000 mm per year in the North, which is mountainous and with few cultivable lands. Consequently, most agricultural activities are undertaken in areas with limited rainfall availability, making irrigation and water management necessary for production (Frija et al., 2009). Around 450,000 ha (8% of useful agricultural land) are irrigated in Tunisia (Chebil et al., 2019), providing 35% of the agricultural production value, 95% of horticultural crops, accounting for 22% of agricultural sector exports and 26% of the total agricultural employment (Frija et al., 2009). Greenhouses in Tunisia cover an area of around 8700 ha, and the major horticultural productions derive from tomatoes (Maaoui et al., 2020 and references therein). During recent decades, the growing demand for irrigation water triggered the implementation of collective irrigation systems, promoting user participation, reformulating the water pricing system and stimulating the adoption of water saving technologies at farm level (Al Atiri, 2004). However, irrigated greenhouse production in the Tunisian "Sahel" region has a low irrigation water use efficiency and does not reflect the water-saving oriented policies that have been applied (Frija et al., 2009). At the moment, farmers specializations and technical efficiency of the greenhouse crop production have a negative correlation with the irrigation water use efficiency, which could be improved by enhancing farmer knowledge through better extension services aiming to ameliorate water resource sustainability and productivity (Frija et al., 2009). During drought, groundwater represents one of the major water resources for irrigation (Zairi et al., 2003), especially in coastal zone of Tunisia, where an intensive greenhouse crop production is present. However, it is important to notice that also the groundwater is vulnerable to quality degradation, due to intensive agricultural and anthropogenic activities, and shows a seasonal variability (Khawla and Mohamed, 2020). In addition to water-related problems, in Tunisia there is also a progressive salinization of soils, which is determining the application of SCS. Interesting experiments, for example, concern the use of substrates deriving from "local wastes", as palm trees compost, compost of oasis wastes, and animal manure, or sand and coconut fibre for tomato production (Elabed and Hadded, 2018). Radhouani and colleagues (2011) performed a trial on muskmelon cultivated in soilless system, using sand, and compost of dry palm compared to perlite. These experiments indicate that local substrates allow obtaining good quality products, opening future scenarios more environmentally friendly.

1.3. Protected cultivation system in Israel and the case of the Arava region

Israel's agricultural sector is characterized by an intensive production due to the need to overcome the scarcity of natural resources, particularly water (Regev, 2006). Over half of the country is indeed characterized by arid or semi-arid areas and only the coastal strip and several inland valleys provide the conditions to vegetables production (Regev, 2006). Israel's annual rainfall ranges from 800 mm in the North of the country to 25 mm on the desert edge in the South, from October to April, and the average annual evaporation ranges from 1,400 mm to 2,800 mm (Azenkot, 2006). Although most of the water resources are located in the North and Central part of the country, agriculture and settlements have also been expanded in the South (Azenkot, 2006). This has been possible thanks to the cooperation and interaction between researchers, extension services, farmers and agro-industries which transformed agriculture into a system that is globally renowned for its efficiency and productivity with extensive protected cultivation systems (Gross, 2006; Regev, 2006; Yurista, 2006). Generally speaking, agriculture in Israel reached a very high technological level (Megersa and Abdulahi, 2015). The total area covered with intensive agriculture (greenhouses, shade-houses, and walk-in tunnels) increased from 900 ha in the 1980s to about 13,000 ha in 2012, despite a slight decrease in the total cultivated area in Israel, with 8,000 ha for vegetables production and 5,000 ha for floriculture (Amir, 2006), and an average farm size of 7 ha (Megersa and Abdulahi, 2015). In addition to vegetable and flower crops that have been grown in greenhouses in the last few decades, fruits such as grapes, pomegranates, and citrus are now grown in plastic or net-houses (Amir, 2006) (Figure 5). Thus, production under protected conditions has become the principal way for Israeli growers to ensure a standard, constant, year-round supply of high-quality products allowing exportation to Europe, taking advantage of local climatic and environmental conditions optimizing the use of land, water and chemicals (Amir, 2006). The advanced greenhouse currently used in Israel includes curtains, skylights and shade netting which move automatically in reaction to sunlight. The structures are 5-m high at their lowest point in order to provide best light and working space, while ventilation is provided by the installation of thermal coverings (Amir, 2006). Many strategies have been implemented in recent years to cool the greenhouses during the day and to increase the temperature at night with a minimal investment of energy (e.g. misting/fogging systems have been tested successfully for vegetables and are used for ornamental plants) (Amir, 2006). Research on irrigation systems is ongoing since the early 1950s and it soon became clear that water use efficiency is much higher using pressurized irrigation system compared to surface irrigation (Azenkot, 2006).

Figure 5. Typical net-house in Israel

Water use efficiency is further increased by implementing automatic valves and computerized controllers, using micro-irrigation systems or vegetal indicators such as leaf water potential and fruit growth rate to achieve further precision and regularity in water and nutrient application (Azenkot, 2006). Drip irrigation represents an efficient method of water distribution also in this area of the world (Trifonov et al., 2017 and references therein).

One of the most important weaknesses of the Israel protected cultivated system is that the use of potable water for agriculture was reduced of about 50% on behalf of an increase of the brackish and reclaimed water not only for irrigation of salinity-tolerant crops such as cotton but also for several crops such as tomatoes and melons (Azenkot, 2006). Approximately 25% of greenhouses with SCS have switched to closed/re-circulating irrigation systems by reusing water drainage either back to the same or nearby field being the most efficient, environmental and economical solution to overcome the problem of drought, and which allowed saving 30-40% of water and fertilizer inputs as well. However, this solution would lead to the rise of microbiological hazards if not extensively treated (Azenkot, 2006). Moreover, the application of aquaponic systems is under study in Israel, although the consumer preferences are still under consideration (Greenfeld et al., 2020). This technique could allow to an increase in the production, and to a reduction of waste and energy and, in most cases, of water usage.

Arava is a region in the Negev desert in the South of Israel characterized by extremely hot and arid climate, strong winds and water shortage (Gadiel, 2006). Local groundwater sources are at a depth of 1,000 m, present high salinity level and a geothermal temperature ranging from 35 to 60°C (Gadiel, 2006). Additional water is obtained from the seasonal flooding of streams and collected in reservoirs after rainfall (average 5-35 mm/year) (Gadiel, 2006). Desert silt soil is formed from settled alluvial materials, is completely deficient of organic matter, infertile, and saline. Consequently, the addition of soil from the Arava riverbed allow obtaining the conditions for growing vegetables such as onions, potatoes, peppers, tomatoes, melons, and eggplants (Gadiel, 2006; Trifonov et al., 2017).

Switching from open fields to greenhouses and net-houses productions allowed producing different varieties of crops with high yields and quality able to successfully compete in the international market. Indeed 60% of Israel`s export of fresh vegetables comes from Arava region (Gadiel, 2006). For example, the major crop cultivated in the Arava Valley during winter is sweet bell pepper. Fallik and colleagues (2019) evaluated, for two consecutive years, the effects of water quantity (irrigation water) and quality (salinity) on pepper yield and fruit quality, also in post-harvest. They concluded that fruits cultivated in greenhouse and irrigated with moderately saline water, of EC 2.8 dS m^{-1}, still maintain a good quality. On the other hand, higher salinity levels significantly affected, in a negative way, both pepper yield and postharvest quality. This reminds us that the cultivation phase is crucial for the final quality of the produce, which is obtained in the field.

1.4. Australian low-cost hydroponic greenhouse for cucumber cultivation

In Australia, as in many other developed economies, irrigated horticulture has increasingly moved from small farm, to large, highly specialised and intensive production systems. This results in a growing demand of water. Agriculture represents 67% of total water consumption in Australia (Hickey et al., 2006). The Australian greenhouse industry is dominated (up to 80%) by low-cost simple structures using TCS, other media or SCS and the hydroponics system for supply water and nutrients to plants (Grewal et al., 2011).

Low-cost greenhouse and hydroponic producers of cucumber and tomatoes have great potential to improve water and nutrient use efficiency because the greenhouse production input re-use is very limited due to the risk in microbial infections or reduced yield due to a non-optimal management of the water and NS (Grewal et al., 2011). Since drainage water contains nutrients, it can in fact contribute to the pollution of local waterways (Thompson et al., 2007). Grewal et al. (2011) reported a study on the opportunities of using the recycle in a commercial farm in Londonderry - New South Wales, Australia. Farm is organized with 18 greenhouse tunnels (12 used to produce cucumbers and 6 to tomatoes), each one with an area of 450 m^2.

The cultivation described in the trial was conducted with a hydroponic system in which the NS (water and minerals) was distributed through drippers to the plants. Plants were growing in black plastic bags filled with a potting mixture. The study showed that in cucumber greenhouse growing system, 38% of the total irrigation water applied was used by the plants and the remaining 62% was discharged from the greenhouse as drainage water. The reuse of the drainage NS resulted in 33% saving of the total potable water for cucumber production and in the reutilization of 566 kg/ha N, 25 kg/ha P and 703 kg/ha K which was used both for cucumber and for other crops, preventing its discharge into the local environment (Grewal et al., 2011).

This case study demonstrates that some relatively simple changes and not too expensive technology in irrigation practices within greenhouse systems can considerably improve sustainability of low-cost hydroponic greenhouses helping to minimise the environmental footprint (Grewal et al., 2011).

2. Temperate Regions

Soilless cultivation is practised in large scale in arid regions, as reported in the previous section, but this technique is not only useful in case of water scarcity, low quality water and/or lack of fertile soils.

In fact, currently, hydroponic cultivation is receiving great attention worldwide thanks to very efficient resources management and to the high yield and quality of food production without forgetting the safety of vegetable produce as well (Tzortzakis et al., 2020). Moreover, this kind of systems can be applied with the aim of cultivating in areas with scarce availability of arable land, or even within big cities/metropolis (Kalantari et al., 2017). We are looking at the diffusion of numerous indoor farms (or vertical farms, or plant factories) in this context (Pennisi et al., 2019 a).

Vertical farms allow growers to obtain good production in small areas, also in multiple layers, with less inputs (mostly water and nutrients). There are examples of successful cultivation of various species including leafy vegetables (Figure 6), aromatic plants, microgreens, saffron, mushrooms (Figure 7), strawberries, among others.

Focusing on water consumption, as reported by several authors, vertical farming allows to a real water saving. As an example, lettuce plants growth with conventional method, require around 250 L/kg/y, while hydroponic system showed an estimated water demand of 20 L/kg/y (Barbosa et al., 2015).

Figure 6. Greenhouse vertical farming for lettuce in SCS in Mexico

Figure 7. Indoor vertical farming for mushroom in Iran

In Italy, trials related to the influence of LED lighting on the resources use of lettuce cultivated indoor were conducted (Pennisi et al., 2019b); results suggest an efficient use of water (WUE up to 75 g FW L^{-1}) with a red: blue ratio of 3. Similar information is provided by experiments on basil (Pennisi et al., 2019 a), that showed the greatest WUE in plants grown under a red: blue ratio of 2 or 3 (average value of 44.5 FW L^{-1}).

SCSs in Italy are also applied in leafy vegetables cultivation to save natural resources and mitigate the problem of nitrate accumulation (Santamaria et al., 2001; 2002; Fontana and Nicola, 2009; Cavaiuolo and Ferrante, 2014) (Figure 8). This issue increasingly attracts the attention of producers, companies, and researchers.

In Italy, and especially in the South, tomato is a very widespread crop used for greenhouse cultivation.

Figure 8. Leafy vegetables (basil) in SCS in Italy

Valenzano and colleagues (2008) compared the cultivation of tomato in soil with two hydroponic systems: (NFT; closed cycle) and on rockwool substrate (open cycle). Regarding WUE, the NFT system allowed minimizing the use of water than other systems. Signore et al. (2016) studied, through the use of a closed soilless system, the way to minimize the environmental impact of tomato plants growth in greenhouse and at the same time reduce the accumulation of ions in the recycled NS. SCSs are also used for strawberry production in open and closed-growing systems. Yield and quality of fruits can be higher especially during the unfavourable seasons. Moreover, the cultivation can be carried out 1-1.5 m above ground, improving the harvesting operations (Nin et al., 2018). Indoor cultivation experiments with different lighting systems showed that blue light provided with LED induced higher yield, without effect on the quality parameters (Nadalini et al., 2017). In strawberry cultivation, as reported in a Romanian trial, SCS could be also an effective method to prevent root diseases (Adela et al., 2013). Aromatic and medicinal plants are other crops that can be grown in hydroponic and aeroponic system, allowing to obtain a greater concentration in bioactive molecules (Hayden et al., 2002; Giurgiu et al., 2015), high yields, and production with minimum residues of pesticide (Rachappanavar et al., 2019). In Romania, studies on basil and mint cultivated in three different hydroponic systems (one horizontal and two vertical) revealed that the considered species were positively influenced by this kind of crop system, in terms of mineral content and nutritional quality, ensuring an optimal use of the resources (Dobrin et al., 2018). In this geographical area, SCSs are also applied for the cultivation of: cucumbers, as reported by Sorin et al. (2015) in a trial on perlite substrate; lettuce plants grown with NFT (Drăghici et al., 2016); tomatoes on mattresses filled with perlite (Drăghici et al., 2013).

CONCLUSIONS

The application of highly specialized farming like greenhouses equipped with SCSs offers the possibility to grow horticultural crops in any kind of environment, including marginal and arid lands that conversely could not be used for agricultural purposes. Moreover, innovative growing systems allow maximizing the yield and extending the growing season due to the possibility to control several environmental factors (i.e. light, temperature, humidity) and to the improvement of nutrients and water use efficiency. Therefore, the economic and environmental sustainability of these intensive cultivation systems is improved. The need for increasing the crop productivity of certain arid and desert areas stimulated the development of innovative and advanced technological solutions that allowed overcome the limitations due to sub-optimal environments. This technological improvement contributed to the creation of a novel perspective and opened new possibilities in crop production, allowing a more rational use of agricultural resources, also in those areas characterized by less challenging environmental conditions.

Today horticulture must face a series of rapid changes in order to adapt to the environmental, economic, and social challenges that the world is experiencing. Thus, the application of highly specialized cultivation techniques can represent an efficient system to increase food security and sustainability in areas where both land, water and crops are scarce as well as in more temperate/favourable regions. New frontiers of SCS application are represented by the indoor vegetables production in urban and peri-urban environments. These applications require an implementation such as the use of ICT for real time crop managements and environmental control. Lighting can be considered a production factor with the possible modulation considering the crop needs during cultivation. In this context, the awareness of growers, supply chain partners, research institutes and governments toward technical and socio-economic aspects of greenhouses and SCSs is fundamental for the future development of horticulture.

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FLOWERS QUALITY IN RELATION TO PLANTING PERIOD IN SOME HYACINTH CULTIVARS

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Abstract

The study evaluated the quality of flowers in nine cultivars of Hyacinthus orientalis L. according to planting time and forcing period. Biological material was represented by nine Hyacinth cultivars: 'Carnegie' (Car), 'Blue Jacket' (B J), 'Blue Star' (B S), 'China Pink' (C P), 'Fondante' (Fon), 'Gipsy Queen' (G Q), 'Pink Pearl' (P P), 'Rembrandt' (Rem), 'Miss Saigon' (M S). A mixture of leaf soil and sand represented the growth substrate. The planting was done on three different calendar dates: November 10 (P I), November 24 (P II), and December 12 (P III) 2018, respectively. Forcing period (FP: FP I, FP II, FP III), flower stem length (FS: FS I, FS II, FS III) and flowering duration (FD: FD I, FD II, FD III) were evaluated. 'Blue Jacket' (B J) cultivar showed the highest values for flower stem (FS), with statistically assured differences for LSD 0.01% (FS I and FS II) and for LSD 5% (FS III), respectively. Differences statistically ensured for LSD5% were also recorded in 'Carnegie' (Car), 'Fondante' (Fon) cultivars (FS I and FS II), and in 'Gipsy Queen' (G Q) cultivar (FS I, FS II and FS III), respectively. The variation of the FS parameter in relation to FP, was described by a polynomial equation of degree 3 under conditions of R^2=0.839, p=0.045 for FP I, and by a polynomial equation of degree 2 under conditions of R^2=0.923, p=0.0016 for FP II. The flowering duration (FD) according to the forcing period (FP) was described by a polynomial equations of degree 2, under conditions of R^2=0.873, p=0.0057 for FP I, and in conditions of R^2 = 0.621, p=0.085 for FP II, respectively. PCA explained 58.498% of variance for PC1 and 30.622% of variance for PC2, in relation to the forcing period (FP); explained 58.052% of variance for PCI, and 34.78% of variance for PC2 in relation to flowering duration (FD), and explained 93.882% of variance for PC1 and 4.1988% of variance for PC2, respectively, in relation to flower stem (FS). Cluster analysis (two way) facilitated the grouping of the cultivars based on affinity in relation to FP, FD and FS, under statistical safety conditions (Coph. corr = 0.849).

Key words: *cluster analysis, flower quality, flowering period, Hyacinth, PCA.*

INTRODUCTION

Hyacinthus orientalis L. (*Asparagaceae*) has its origin in the Eastern part of the Mediterranean region. From Turkey it was introduced to Eastern Europe and a number of genotypes were improved to obtain and grow varieties with new decorative qualities (Hosokawa, 1999). During the Victorian period, there were about 2000 varieties in cultivation, and at present, it is estimated that 187 are registered in the International Register (Stebbings, 1996; Hosokawa, 1999), of which about 70 are most commonly cultivated as garden flowers, or in pots. The varieties differ by the size of the plants, the color of the flowers (white, yellow, pink, red, purple, blue), the type of petals (single or double), perfume. Anthocyanin compounds are an important factor for flower color and over 20 anthocyanins have been identified and isolated in hyacinth flowers (Harborna, 1964; Hosokawa et al. 1995a-c, 1996a,b, 1999).

It is a species cultivated for large-scale ornamental purposes in the Northern Hemisphere (Hosokawa, 1999; Nazari et al., 2011; Souza and Lorenzi, 2012; Alexandre et al., 2017). Individual flowers, as cut flowers, can also be use in indoor flower arrangements for different festive times, or for obtaining essential oils in the perfume and cosmetics industry (Hosokawa and Fukunaga, 1995; Nazari et al., 2011).

The importance of some species in ecosystem (Patoka et al., 2016; Jones et al., 2018), relationships with cultivation conditions (Addai, 2011; Smigielska et al., 2014; Karagöz et al., 2019), sensitivity to pathogens, genetic transformations in cultivated varieties and transmission of pathogens through propagation methods (Koetle et al., 2015; Patoka et al., 2016; Alexandre et al., 2017), the potential for transfer of pathogens to the international flower market (Santos et al., 2006; Çiğ and Başdoğan, 2016; Patoka et al., 2016) and obtaining forms resistant to pathogens (Popowich et al., 2007), have been analyzed by numerous studies and researches.

The methods, conditions and factors that influence the propagation of *Hyacinthus* genotypes, and bulblet induction (Krause, 1980; Cheesman et al., 2010), as well as the relationship with the growth substrates (Nazari et al., 2011), or the response to stress conditions (Türkoglu et al., 2011; Koksal et al., 2014) were studied.

Providing optimal conditions during the forcing period in relation to *Hyacinthus* varieties is very important, so the influence of factors such as temperature, humidity, light etc., have been studied on the evolution of plants and on the flowers quality (Dole, 2003; Addai and Scott, 2011). Flowers quality depends on the genotype and a number of factors such as forcing period, vegetation conditions (temperature, light through spectrum and intensity, growth medium etc.) (Nazari et al., 2011; Śmigielska et al., 2014).

The present study evaluated the influence of planting date and forcing period on flower quality in nine cultivars of *Hyacinthus orientalis* L.

MATERIAL AND METHODS

The study evaluated the quality of flowers in nine cultivars of *Hyacinthus orientalis* L. according to the time of planting and the forcing period. Biological material was represented by nine cultivars: 'Carnegie' (Car), 'Blue Jacket' (B J), 'Blue Star' (B S), 'China Pink' (C P), 'Fondante' (Fon), 'Gipsy Queen' (G Q), 'Pink Pearl' (P P), 'Rembrandt' (Rem), 'Miss Saigon' (M S).

The bulbs showed a high degree of uniformity in the case of each cultivar: 82.2 ± 1.38 g at 'Carnegie' (Car), 79.6 ± 1.43 g at 'Blue Jacket' (B J), 64.6 ± 1.29 g at 'Blue Star' (B S), 77.2 ± 1.98 g at 'China Pink' (C P), 77.4 ± 1.83 g at 'Fondante' (Fon), 87.4 ± 1.91 g at 'Gipsy Queen' (G Q), 63.1 ± 1.09 g at 'Pink Pearl' (P P), 73.8 ± 1.2 g at 'Rembrandt' (Rem), and 67.2 ± 1.95 g at 'Miss Saigon' (M S), respectively.

A mixture of leaf soil and sand represented the growth substrate, growt medium suitable for Hyacinth (Sala, 2011).

The planting was done on three different calendar dates: first planting (P I) on 10 November; second planting (P II) on 24 November and third planting (P III) on 8 Deccember 2018. The duration of the forcing period (FP), from the time of planting to flowering, was evaluated. Depending on the date of planting, three forcing periods were recorded: FP I in case of P I, FP II in case of P II and FP III in case of P III.

In order to evaluate the flowers quality, the length of the flower stem (FS) and the flowering duration (FD) were determined for the nine cultivars, in relation to the planting date and the forcing period.

To obtain the values for limit of significance of differences (LSD) the analysis of variance was used. In order to evaluate the degree of interdependence between the variables studied (FP, FD, FS), the correlation analysis was used. The behavioral models of some variables, as well as the statistical safety coefficients, were obtained by regression analysis. Principal Component Analysis and Cluster Analysis were used to evaluate the level of variance according to the main components, as well as to obtain the classification and association of the experimental variants. For the statistical certainty of the results, the correlation coefficients r and R^2, and p and F parameters were used. For the statistical analysis and processing of the experimental data, EXCEL and PAST programmes were used (Hammer et al., 2001).

RESULTS AND DISCUSSIONS

Depending on the time of planting (P I, P II, P III) the plants within the nine cultivars have undergone different periods of forcing (FP I, FP II, FP III) until the time of flowering. In the case of P I planting, there was a forcing period between 69 days in the 'Fondante' (Fon) and 'Gipsy Queen' (G Q) cultivars, and 76 days in the 'Rembrandt' (Rem) cultivar. For P II planting, the forcing period ranged from 85 days in 'Fondante' (Fon) cultivar and 89 days in 'Blue Jacket' (B J) and 'Gipsy Queen' (G Q) cultivars. In the case of P III planting, the forcing period varied between 83 days in the 'Carnegie' (Car) cultivar and 94 days in the 'Fondante' (Fon) cultivar (Table 1).

As a result of the planting times and the forcing period, three flowering durations (FD) were recorded (FD I, FD II and FD III). Flowering duration FD I varied between 10 days in 'China Pink' (C P) cultivar and 21 days in 'Gipsy Queen' (G Q) cultivar. Flowering duration FD II varied between 19 days in 'Blue Star' (B S) cultivar and 28 days in 'Blue Jacket' (B J) cultivar. Flowering duration FD III varied between 5 days in 'China Pink' (C P) cultivar and 18 days in 'Miss Saigon' (M S) cultivar (Table 1).

The dimensions of the flower stems (FS) varied according to cultivar, time of planting and forcing period (Table 2).

Table 1. Forcing period (FP) and flowering duration (FD) depending on the date of planting

Hyacinth cultivars	Values for forcing period (FP) and flowering duration (FD) depending on the date of planting					
	P I (10 11 2018)		P II (24 11 2018)		P III (8 12 2018)	
	FP I	FD I	FP II	FD II	FP III	FD III
	(days)					
'Carnegie' (Car)	71	18	88	26	83	14
'Blue Jacket' (B J)	75	19	89	28	90	16
'Blue Star' (B S)	73	14	85	19	92	11
'China Pink' (C P)	74	10	87	21	91	5
'Fondante' (Fon)	69	20	85	27	94	11
'Gipsy Queen' (G Q)	69	21	89	23	91	8
'Pink Pearl' (P P)	72	13	86	20	86	14
'Rembrandt' (Rem)	76	13	86	22	89	10
'Miss Saigon' (M S)	74	13	88	22	88	18

P – time of planting (P I – first planting; P II – second planting; P III – third planting); FP – forcing period; FP I – forcing period for first planting (P I); FP II – forcing period for second planting (P II); FP III – forcing period for third planting (P III); FD – flowering duration; FD I – flowering duration I (associated with P I and FP I); FD II – flowering duration II (associated with P II and FP II); FD III – flowering duration III (associated with P III and FP III)

Table 2. Size of the flower stem in the Hyacinth cultivars studied, in relation to the forcing period

Hyacynth cultivars	Floral stem length (cm) depending on the date of planting								
	P I (10 11 2018)			P II (24 11 2018)			P III (8 12 2018)		
	FS I Mean values	Diferences	Signification	FS II Mean values	Diferences	Signification	FS III Mean values	Diferences	Signification
'Carnegie' (Car)	11	1.67	*	10.66	0.89	-	8.33	-0.32	-
'Blue Jacket' (B J)	12.33	3	***	13.66	3.89	***	11	2.34	*
'Blue Star' (B S)	7.33	-1.99	0	7.33	-2.44	0	7.66	-0.99	-
'China Pink' (C P)	7.66	-1.66	0	8.66	-1.11	-	8.33	-0.32	-
'Fondante' (Fon)	11.33	2	*	11	1.33	-	10	1.34	-
'Gipsy Queen' (G Q)	11	1.67	*	11.66	1.89	*	10.66	2	*
'Pink Pearl' (P P)	8	-1.33	-	7.33	-2.44	0	6.66	-1.99	0
'Rembrandt' (Rem)	7	-2.33	0	8	-1.77	0	7.66	-0.99	-
'Miss Saigon' (M S)	8.33	-0.99	-	9.66	-0.11		7.66	-0.99	-
Control	9.33			9.77			8.66		
Limit of significance of differences (LSD)	LSD5%=1.53; LSD1%=2.10; LSD0.1%=2.86			LSD5%=1.54; LSD1%=2.12; LSD0.1%=2.88			LSD5%=1.83; LSD1%=2.51; LSD0.1%=3.42		

FS I – flower stem in P I conditions; FS II – flower stem in P II conditions; FS III – flower stem in P III conditions

The quality of the flowers, based on the size of the flower stem (FS), a parameter that varied in relation to forcing period (FP), was evaluated. Larger flowers have higher quality and are more appreciated. The values for flower stem (FS I) ranged between 7.00 cm in 'Rembrandt' (Rem) cultivar and 12.33 cm in 'Blue Jacket' (B J) cultivar, in the case of FP I forcing period. The length of the flower stem (FS II) was between 8.00 cm in ‘Rembrandt’ (Rem) cultivar and 13.66 cm in ‘Blue Jacket’ (B J) cultivar in the case of FP II forcing period. The floral stems (FS III) had dimensions between 6.66 cm in ‘Pink Pearl’ (P P) cultivar and 11.00 cm in ‘Blue Jacket’ (B J) cultivar in the case of FP III forcing period.

The ‘Blue Jacket’ cultivar presented the highest values for the flower stem in all three flowering periods, with statistically assured differences for LSD 0.01% (P I and P II planting period) and LSD 5% (P III planting period). ‘Carnegie’, ‘Fondante’ (P I and P II planting periods) and ‘Gipsy Queen’ cultivars (P I, P II, P III planting periods) were also evidenced.

In the case of the first planting (P I), the variation of flower stem length, depending on the forcing period (FP I), was described by a polynomial equation of degree 3, relation (1), under conditions of $R^2 = 0.839$, $p = 0.045$.

The graphical distribution of the FS parameter values according to the forcing period (FP), for the planting variant P I, is presented in Figure 1.

$$y = 0.02207x^3 - 4.746x^2 + 339.3x - 8056 \qquad (1)$$

where: y - flower stem - FS I (cm); x - forcing period - FP I (days).

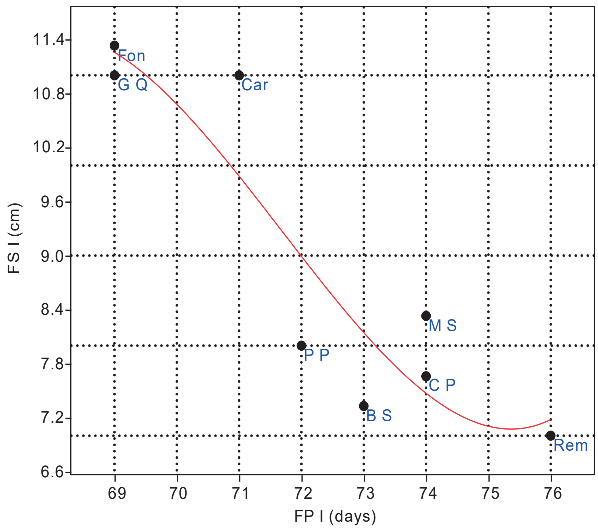

Figure 1. Flower stem (cm) in relation to forcing period (FD I) in condition to P I planting
(analysis without ‘Blue Jacket’ cultivar)

In the case of P II plantations, the length of the flower stem (FS) according to the forcing period (FP II) was described by a polynomial equation of degree 2, relation (2), in conditions of $R^2=0.923$, $p=0.0016$. The graphical distribution of the FS parameter values, according to the forcing period (FP II), for the planting variant P II, is presented in Figure 2.

$$y = 0.3544x^2 - 60.36x + 2577 \qquad (2)$$

where: y - flower stem - FS II (cm); x - forcing period - FP II (days).

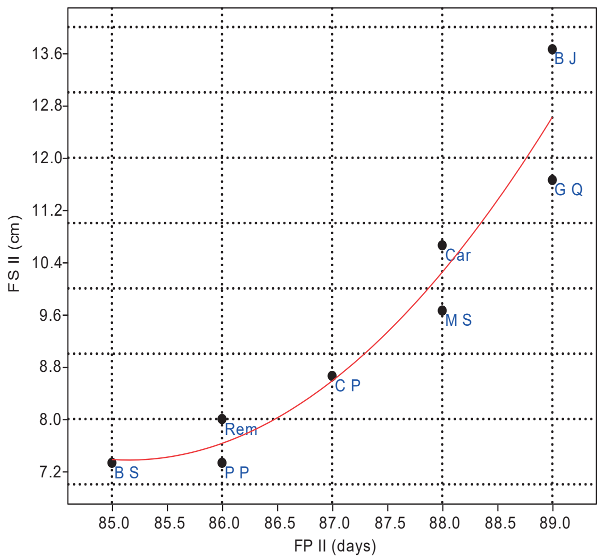

Figure 2. Flower stem in relation to forcing period in condition of P II planting
(analisys without ‘Fondante’ cultivar)

The length of the flower stem presented a high variability in relation to forcing period FP III (CV = 17.5502). As a result, the analysis of the data did not lead to obtaining a model of variation of the parameter FS III in relation to FP III, under statistical safety conditions.

Flowering duration also has a high importance regarding the quality of flowers, both from a practical and economic point of view.

For the first planting period (P I) the highest flowering duration was recorded in the ‘Gipsy Queen’ (G Q) cultivar (21 days), followed by ‘Fondante’ (Fon) (20 days), ‘Blue Jacket’ (B J) (19 days) and ‘Carnegie’ (Car) (18 days), and the shortest period of flowering was recorded at ‘China Pink’ (C P) (10 days).

In case of P II planting, the highest values of flowering duration were recorded in the ‘Blue Jacket’ (B J) cultivar (28 days), followed by ‘Fondante’ (Fon) (27 days), and ‘Carnegie’ (Car) (26 days), and the lowest value was recorded in the ‘Blue Star’ (B S) cultivar (19 days). In the case of P III planting, the highest flowering duration was recorded in the ‘Miss

Saigon' (M S) cultivar (18 days) followed by 'Blue Jacket' (B J) (16 days), the other cultivars having shorter flowering durations, and the shortest was at 'China Pink' (C P) (5 days). The comparative analysis regarding the flowering duration for the three planting periods, highlighted the P II planting period during which the highest flowering duration in the studied cultivars was recorded. The total flowering duration (TFD) calculated as $TFD = FDI + FDII + FDIII$, led to the graphical representation in Figure 3.

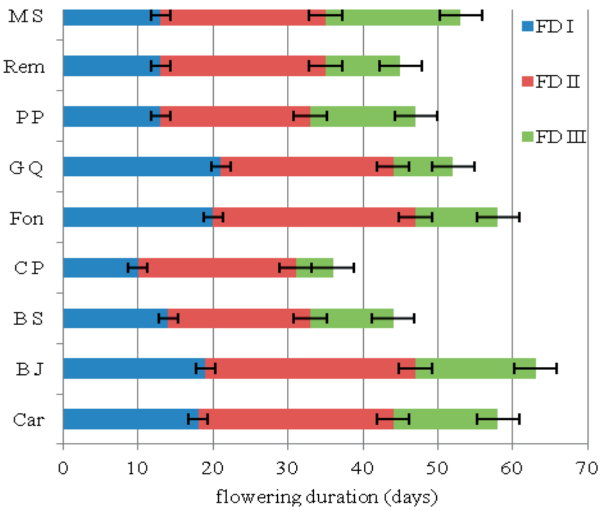

Figure 3. TFD diagram for Hyacinth cultivars according to planting period

In relation to TFD, there were four classes of 10 days each. In class C1 (TFD = 30-40 days) C P cultivar was classified; in class C2 (TFD = 40-50 days) B S, P P and Rem cultivars were included; in class C3 (TFD = 50-60 days) Car, Fon, G Q and M S cultivars were classified, and in class C4 (TDF> 60 days) the B J cultivar was included.

The possibility of flowering duration (FD) estimating was tested according to the forcing period (FP), variable that depended on the planting times (P I, P II, P III).

In the case of the P I planting time, estimation of flowering duration (FD I) was possible based on a polynomial equation of degree 2, relation (3), under conditions of $R^2 = 0.873$, p = 0.0057, graphical distribution being presented in Figure 4.

$$y = 0.2466x^2 - 36.97x + 1397 \qquad (3)$$

where: y - flowering duration - FD (days); x - forcing period - FP (days).

For the P II planting variant, the flowering duration (FD II) according to the FP II forcing period was described by a polynomial equation of degree 2, relation (4), under conditions of $R^2 = 0.621$, p = 0.085, graphical distribution being presented in Figure 5.

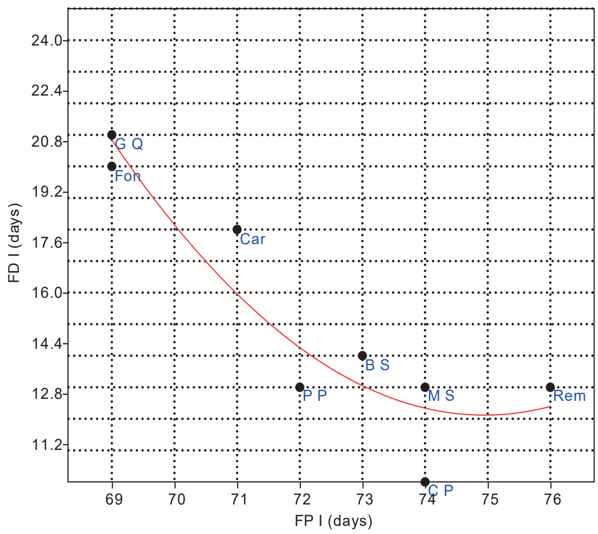

Figure 4. Graphical distribution of flowering duration (FD) according to the forcing period (FP), in case of P I planting variant

$$y = 0.08503x^2 - 13.22x + 528.7 \qquad (4)$$

where: y - flowering duration - FD (days);
x - forcing period - FP (days).

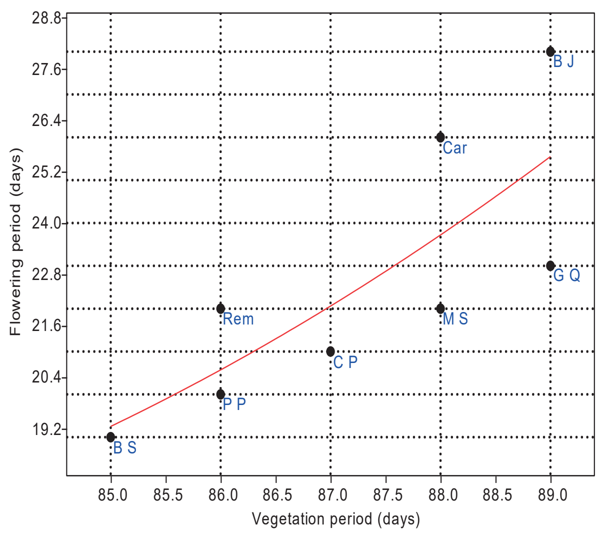

Figure 5. Graphical distribution of flowering duration FD II according to the FP II forcing period, in case of P II planting variant

For the planting period P III, the flowering duration (FD III) showed a high variability (CV = 34.0224), and by analyzing the data in relation to the FP III forcing period, a model describing the FD III variation in relation to FP III was not obtained under statistical safety conditions.

PCA was used to evaluate the distribution and association of cultivars in relation to the three forcing periods (FP I, FP II, FP III), flowering durations (FD I, FD II, FD III), and flower stem (FS I, FS II, FS III). PCA analysis explained 58.498% of variance for PC1 and 30.622% of variance for PC2, in relation to the forcing period (FP), Figure 6.

PCA diagram (Figure 6) show how the cultivars M S, Rem and B J were associated with the biplot FP I; Carp and P P cultivars were associated with FP II biplot, and C P and B S cultivars were associated with FP III biplot.

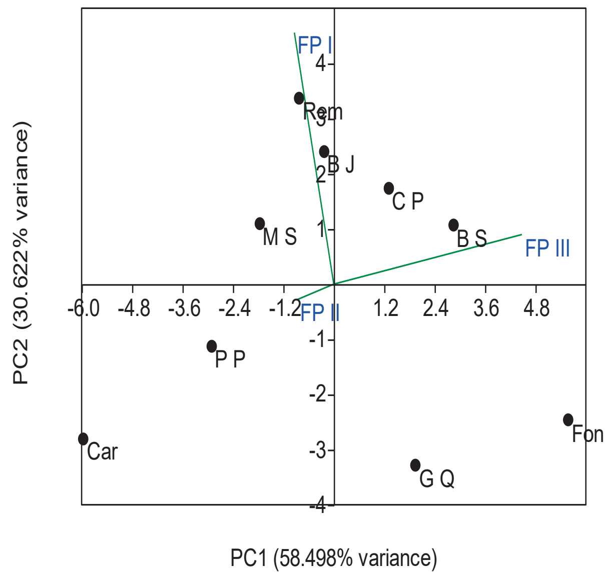

Figure 6. PCA diagram in relation to forcing period (FP) for Hyacinth cultivars studied

In relation to flowering duration (FD), PCA explained 58.052% of variance for PCI, and 34.78% of variance for PC2. From the PCA diagram shown in Figure 7, were found the association of G Q and Fon cultivars with FD I and FD II biplots, respectively the association of M S, Car and B J cultivars with FD III biplot.

In relation to flowers stem (FS), PCA explained 93.882% of variance for PC1 and 4.1988% of variance for PC2, respectively. From the PCA diagram shown in Figure 8, were found the association of Car and Fon cultivars with FS I biplot, and the association of G Q and B J cultivars with FS II and FS III biplots.

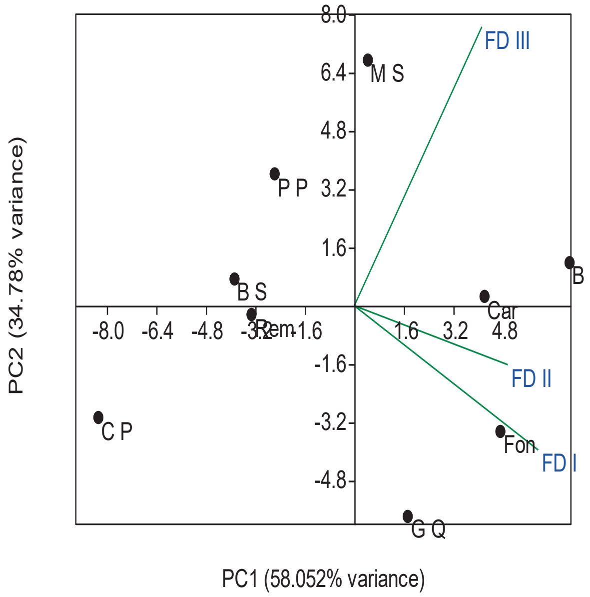

Figure 7. PCA diagram in relation flowering duration (FD) for Hyacinth cultivars studied

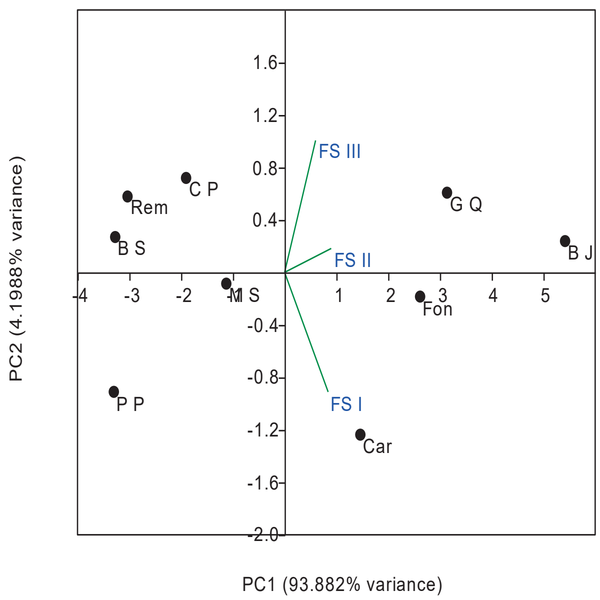

Figure 8. PCA diagram in relation to flowers stem (FS) for Hyacinth cultivars studied

Cluster analysis (two way) facilitated the grouping of cultivars on the basis of affinity with respect to the forcing period (FP) and flower stem (FS), under statistical safety conditions, Coph. corr. = 0.849 (Figure 9).

From the dendrogram analysis shown in Figure 9, were found high affinity between FP II and FP III (common subcluster) and high affinity between FS I and FS II (common subcluster).

Regarding the grouping of cultivars in relation to the two parameters (FP and FS), two distinct clusters were formed with several sub-clusters each. In the CI cluster, 'Fondante' (Fon) and

'Gipsy Queen' (G Q) cultivars were grouped with similar results (common subcluster), and 'Blue Jacket' (B J) cultivar being associated with the respective sub-cluster.

In C2 cluster, two sub-clusters with high affinity (B S and C P, respectively Rem and M S) were identified, to which the P P cultivar is attached, followed by the Car cultivar.

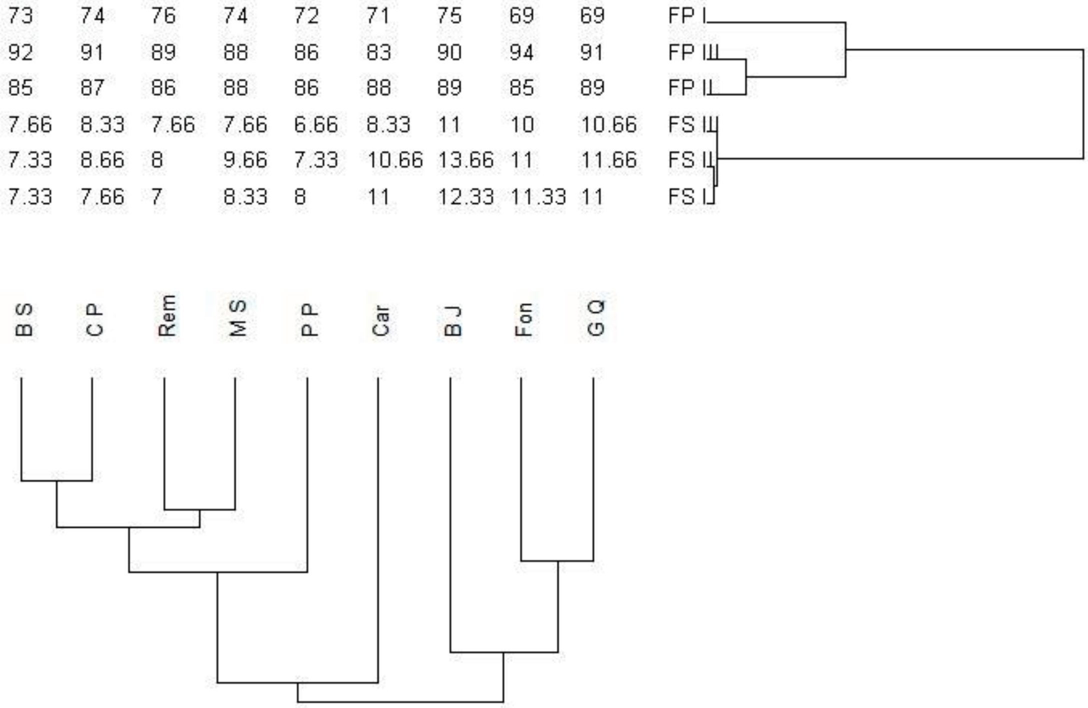

Figure 9. Dendrogram based on Cluster analysis, two way, on the nine Hyacinth cultivars in relation to FP and FS

Ornamental plants are important both by the decorative effect and by the functional role at the level of natural or urban ecosystem (Baiyewu et al., 2005; Acar et al., 2007; Mohamad et al., 2013; Dragoș et al., 2018).

For the qualitative appreciation of the ornamental plants through the leaves, a series of elements such as the shape, dimensions and spatial distribution of the leaves are important (Di Benedetto et al., 2006; Li et al., 2014; Santagostini et al., 2014).

In addition, the leaf surface, for which different estimation methods were elaborated (Sala et al., 2015; Drienovsky et al., 2017; Cândea-Crăciun et al., 2018), the health and intensity of the leaf color (Kim et al., 2012; Bayat et al., 2018), are parameters which, moreover, contributes to the plants functioning, and in decorative and contrasting effect of ornamental plants.

For ornamental plants through flowers, morphological description and qualitative assessment was made on the basis of parameters, descriptors and qualitative indices of flowers (shape, size, color, flowering duration, perfume etc.), as well as the flowers/leaves proportion and balance, and the plants as whole (Dobrilovič, 2010; Kuligowska et al., 2015; Huss et al., 2018).

The quality of the flowers at Hyacinth was evaluated based on several parameters that concern the plant as a whole. It presented interest especially the height of the plant, the length of the inflorescence, the length of the leaves, the diameter and the circumference of the inflorescence, the number of flowers in inflorescence, the petals arrangement, flowering duration, color, perfume (Addai, 2011; Śmigielska et al., 2014; Çiğ and Koçak, 2019).

Flowers dimensions (length of the flower stem, diameter, circumference) and the number of inflorescences were studied and appreciated as important elements in the qualitative assessment at Hyacinth (Addai, 2011).

The colors of leaves and flowers in plants in general, and especially in ornamental plants have been intensively studied and have been found to be of particular importance for the management of species and genotypes (Kendal et al., 2013; Zhao and Tao, 2015; Noman et al., 2017).

Flower color is genotype specific, and anthocyanin profiles were studied in flower petals in different genotypes of *Hyacinthus orientalis* and grape hyacinth (*Muscari* ssp.) (Tao et al., 2015; Lou et al., 2017). At the same time, the color of the flowers is influenced in intensity, by the pH of the soil or the growth substrate, and certain mineral salts (Sala, 2011, 2018; Yang et al., 2012; Zhao and Tao, 2015).
The perfume complements the spatial architectural ensemble and creates a special atmosphere through elegant notes (Fenske and Imaizumi, 2016; Noman et al., 2017).
Flowering duration is also important in *Hyacinthus* and has been studied in relation to planting periods, forcing periods, genotypes, growing conditions, mycorrhizal relationships and so on. (Śmigielska et al., 2014; Xie and Wu, 2017). The estimation of the flowering period and duration, has already been performed in other ornamental species, based on polynomial or smoothing spline models (Băla et al., 2018).
The prolonged stress induces disturbances of physiological indices in the ornamental plants and the reduction of the anthocyanin content, which affects the leaves and flowers colors of the ornamental plants (Li et al., 2009; Zhao and Tao, 2015).
Alterations of physiological indices and flower quality in Hyacinthus were recorded under alkaline pH conditions and high concentrations of mineral salts, such as Na (Türkoglu et al., 2011; Koksal et al., 2014).
The results communicated are in accordance with the data and the results presented by the specialized literature that was the basis for the documentation of this study.

CONCLUSIONS

The nine cultivars of *Hyacinthus orientalis* L. had different behavior in terms of vegetation period (VP), flowering duration (FD) and flower stem (FS) as a flower quality element, under similar growing conditions. 'Blue Jacket' (B J) was differentiated by the largest size of the flower stem, followed by 'Fondante', (Fon) 'Carnegie' (Car), and 'Gipsy Queen' (G Q), with statistically assured differences from the control.
Models of polynomial equations of degree 2 and 3 were obtained, to estimate the size of the flower stem (FS) and the flowering duration (FD) in relation to the vegetation period, under statistic safety conditions.
Principal Component Analysis and Cluster Analysis approaches explained the variance and the association of the cultivars in relation to variables studied, under statistical safety conditions.

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COMPARATIVE ANALYSIS OF REGIONAL PARK DENDROFLORAS OF UKRAINE IN THE ZONAL ASPECT

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Abstract

The paper provides the results of the comparative analysis of similarity of the model dendrofloras of protected parks-monuments of landscape art of Rivne, Vinnytsia and Zaporizhzhia administrative regions of Ukraine, the natural conditions of which represent three geographical zones - mixed forests, forest-steppe and steppe. The basis for the comparison was the park dendrofloras of Rivne region inventoried by the authors, which includes 127 species of woody plants. The species composition of plants of park dendrofloras of the other two regions has been analyzed by literary sources, from which it is known that the park dendroflora of Zaporizhzhia region has 348 and Vinnytsia region - 487 species of woody plants. As a result of the pairwise comparison of dendrofloras the greatest similarity of species composition of woody plants of parks of Vinnytsia and Zaporizhzhia regions (Ksc = 0.47, Kj = 0.30) has been established, which is a natural phenomenon, since the richness of forest steppe and steppe soils also determines the richness of dendrodiversity. As a result of the comparative analysis of taxonomic structures of all three park regional dendrofloras, the predominance of Magnoliophyta and Rosaceae species has been established. In their biomorphological structure, a subset of mesophanerophytes with a dominance of the first magnitude trees and tall shrubs has a key place. In the ecological structure of these three dendrofloras there are more light-demanding, mesophytyc and frost-resistant plant species. As for the soil nutrient requirements, a share of common species for the pair of park dendrofloras of Rivne and Zaporizhzhia regions dominates. It is explained geographically and edaphically by less optimal conditions for plant growth and development than in the forest-steppe zone. Also, there are more dendroexotic plants in this pair of compared dendrofloras.

Key words: *woody plants, floral structure, similarity coefficient, natural-geographical zones.*

INTRODUCTION

According to the International Environmental Law, biodiversity conservation is an extremely pressing global issue, as evidenced by a number of conventions and other international instruments. In this aspect, for the conservation of flora, international special purpose policy papers have been developed and implemented, including the European Plant Conservation Strategy, prepared jointly by the Council of Europe and Planta Europa (Pruhonice, 2001), Global Strategy (Global…, 2004) for Plant Conservation (The Hague, 2002), International Agenda for Botanic in Conservation prepared by the Botanic Gardens Conservation International during 1998-2000, and others.

Among the means of implementing the abovementioned documented decisions is the formation by the States of a network of conservation areas for preservation of flora *in situ* and *ex situ*. The legislation of Ukraine on the Nature Reserve Fund (1992), which identifies 11 categories of territories and objects, is also used to accomplish this task. Of these, there are four categories of artificial conservation parks (botanical gardens, dendrological parks, zoological parks, parks - monuments of landscape art). There is the biggest number of parks-monuments of landscape art in Ukraine (89 sites of nationwide and about 460 sites of local importance). Of the nationwide objects network, about 60% of ancient parks, which largely combine autochthonous and introduced dendroflora, formed over the centuries as a separate phytosystem.

Successful scientifically balanced conservation of park dendrofloras requires comprehensive research, including comparative studies to determine the degree of botanical value of the conservation objects, the enrichment of the species composition of plants and their humane use, in particular in the social sphere. Comparative studies are also needed to develop the science of woody plants, the practices of their introduction and conservation. In this regard, the geographical space of Ukraine allows interzonal comparative studies of local protected dendrofloras of different regions. According to the results of the research, the process of replenishing the collections of dendrofloras of artificial protected parks can be adjusted.

As known, dendrological studies are among the priorities, as evidenced by numerous publications (Loeb, 2006; Hassan et al., 2019; Kolodziejczyk et al., 2019). Therefore, the study of dendrofloras of the parks in different regions and the comparison of their species composition of plants are now relevant and important for science and practice. Similar studies are particularly common to European scientists. Amongst other things, dendroflora of different parks has been studied by the European scientists, for example in Romania – Onete et al. (2010), Croatia - Tafra et al. (2012), Poland - Dudkiewicz et al. (2015), Bulgaria - Tashev and Tsavkov (2016) and many others.

Over the last decade, in Ukraine, various aspects of dendroflora of the protected parks or individual regions have been studied in the Steppe zone - Popova et al. (2007), Chonhova (2013), Vlasenko (2016), in the Carpathians - Mykhailovych (2014), in Polissia - Kurdyuk et al. (2015), Markov (2015), Savoskina (2016), Pokotylova (2018), Forest-Steppe - Syplyva (2014), zone of Deciduous Forests - Kotsun (2007), Miskevych (2018) and many other scientists.

At the same time, an integral scientific direction in the conservation of dendrodiversity (dendrosozology), the objects of which are all groups of rare species of woody plants (autochthonous and introduced of protected and unprotected soil) and protected dendrocenoses, has been distinguished in Ukraine. The priority chosen to investigate this category of the rare dendrodiversity for its *in situ* inventory within the natural reserve fund was natural and necessary, as the dendrorarities are, for the most part, a determining component of the intensively degrading in Ukraine and in Europe in general (*Quercion roboris*, *Quercus robur* L.: IUCN Red List) and (*Pinion sylvestris*, *Pinus sylvestris* L.: IUCN Red List) forest and steppe shrub phytocoenoses, as well as oligotrophic marshes listed in the Berne Convention. On the other hand, a tendency is observed *ex situ* to impoverishment of collections of rare dendroexotics of artificial protected parks of Ukraine. Therefore, it was important for the Ukrainian scientists to know the current state of preservation of these dendrorarities. As a result of the development of this line of research, a number of scientific works have been published, primarily monographs (Popovych et al., 2010, 2013, 2017, 2019; The dendrosozological the catalogue of natural-reserved fund of the Forest-steppe of Ukraine, 2011; The dendrosozological the catalogue of natural-reserved fund of the Steppe of Ukraine, 2014; The dendrosozological the catalogue of natural-reserved fund of Ukrainian Polissya, 2017 etc.) and educational publications (Popovych et al., 2009, 2011), which testify to the results of the initial inventory of the protected rare dendrodiversity of the Forest-Steppe, Steppe and Polissia of Ukraine.

MATERIALS AND METHODS

Objective: to carry out a comparative analysis in the zonal aspect of the degree of similarity of structures of regional dendroflora of parks-monuments of landscape art in Rivne region (zone of mixed forests) with south-facing similarly structured dendrofloras of the same category of the nature reserve fund of model-selected Vinnytsia (Forest-Steppe) and Zaporizhzhia (Steppe) regions (Figure 1).

The object of research: structures of regional (representatively selected) dendrofloras of parks-monuments of landscape art of Rivne, Vinnytsia and Zaporizhzhia regions of Ukraine. The research was based on the published lists of dendroflora species.

In a course of the route-field researches for parks-monuments of landscape art of Rivne region we compiled separately for each of these objects and a general regional list of plant species of dendroflora. It was used as the basis for floristic analysis (Pokotylova, 2018) made by the methods of Tolmachov (1986).

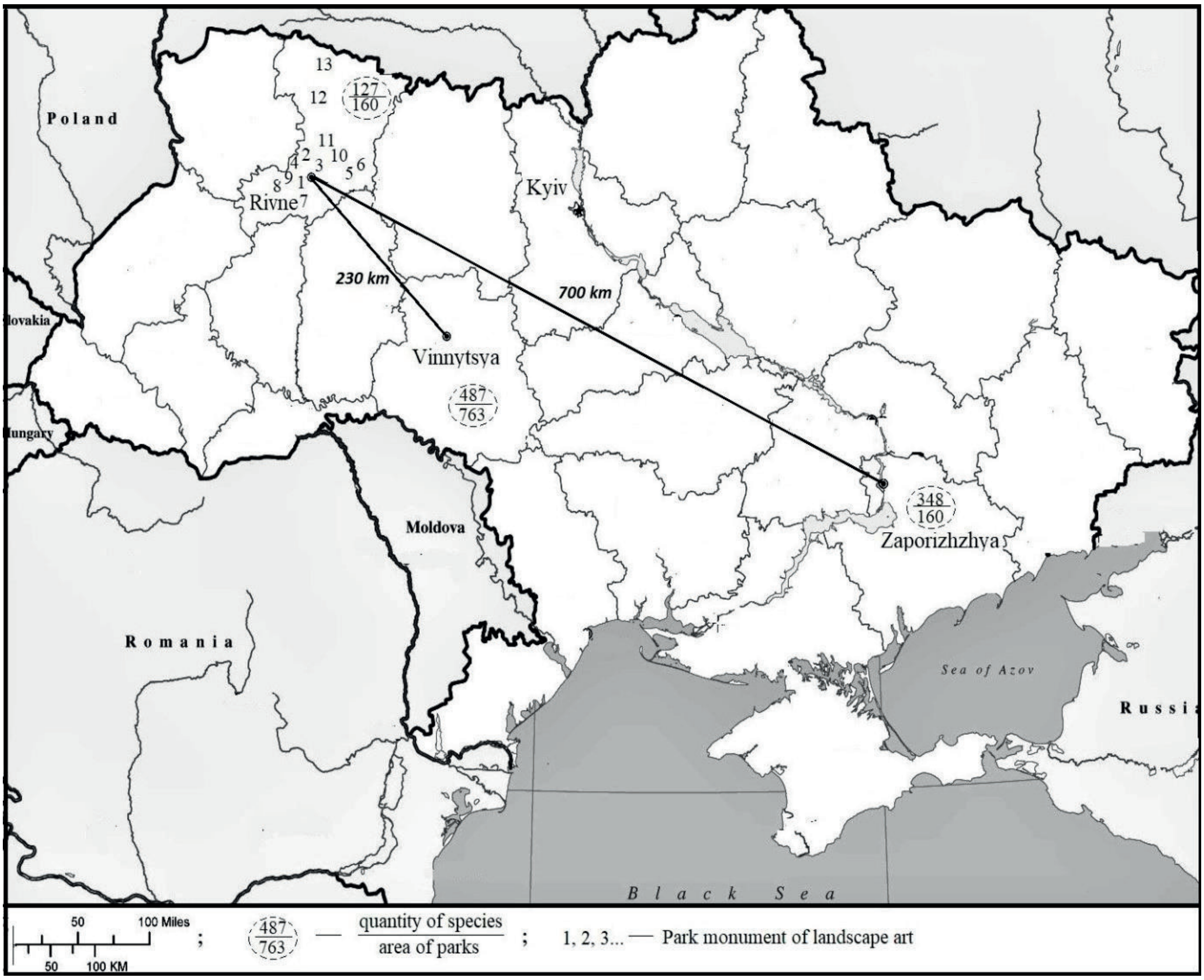

Figure 1. Network of the objects of research on the map of Ukraine

Note: park names: 1 - Rivne Park, 2 - Klevansky Park, 3 - Horodotskyi Park, 4 - Novostavskyi Dendropark, 5 - Hoshchanskyi Park, 6 - Velykomezhyritskyi Park, 7 - Mizotskyi Park, 8 - Mlynivskyi Park, 9 - Ostrozhetskyi Park, 10 - Tuchynskyi Park, 11 - Oleksandrivskyi Park, 12 - Antonivka Park, 13 - Zirnenskyi Park.

We have used similar results for comparison from the studies of dendroflora of the parks-monuments of landscape art of Vinnytsia (Popovych et al., 2012; Syplyva, 2014) and Zaporizhzhia (Chonhova, 2013) regions.

The analysis of taxonomic and ecological structures of dendrofloras was performed on the basis of the methodology used in similar dendrofloristic studies (Popovych et al., 2010; 2011; 2013; 2017; 2019). Traditional environmental analysis of dendroflora was supplemented by the division of plant species into eco-groups relative to the selected ecofactor (Didukh, 1994; Didukh, Pliuta, 1994). The analysis of biomorphological structure was carried out according to the classifications of types of life forms (Raunkiaer, 1937; Serebryakov, 1962; Kalinichenko, 2003). To analyze the geographical structure, we used the botanical-geographical zoning of the Earth for autochthonous plant species (Meusel et al., 1965, Meusel et al., 1978), and for dendroexotic plants we determined their belonging to the floral regions of the Earth (Takhtadzhian, 1978).

We performed a correlation analysis in three stages: 1) calculation of the Serensen-Chekanovsky Ksc coefficient of similarity, 2) calculation of the Jacquard similarity coefficient, 3) comparison of the obtained results (Schmidt, 1984).

Latin names of plant species have been verified with the taxonomic base The Plant List.

RESULTS AND DISCUSSIONS

There are 13 parks-monuments of landscape art (Figure 1) on the area of 160.4 hectares in the territory of Rivne region, where 127 species of woody plants have been found. Of their total number, the vast majority are *Magnoliophyta* species - 81.10% (Table 1).

Table 1. Taxonomic structure of dendroflora of parks-monuments of landscape art in Rivne region

Division	Number of families		Number of genuses		Number of species	
	pcs	%	pcs	%	pcs	%
Pino-phyta	3	10.34	8	11.76	24	18.90
Magno-liophyta	26	89.66	60	88.24	103	81.10
Total	29	100	68	100	127	100

The number of species of dendroflora of the parks - monuments of landscape art of Zaporizhzhia region is much higher - 348 species of plants. They represent 155 genuses, 62 families, two divisions. *Magnoliophyta* species (81.32%) also prevail in Zaporizhzhia region (Chonhova, 2013). The studied parks of Vinnytsia region are filled with the largest number of taxons - 487 species of woody plants, and the main share belongs to *Magnoliophyta* (87.30%) as well (Popovych et al., 2012; Syplyva, 2014).

As a result of the correlation analysis it was established that the value of the Serensen-Chekanovsky similarity coefficient for the local park dendrofloras of the three administrative regions of Ukraine is within 0.36 ≤ Ksc ≤ 0.47, the average value ΔKsc = 0.42. The closest correlation relationships are between the dendroflora of Vinnytsia and Zaporizhzhia Parks (Ksc = 0.47), which is a natural phenomenon, since the richness of soils and the similarity of climatic conditions of the Forest-Steppe and Steppe of Ukraine determine both quantitative and qualitative richness of the dendrodiversity. In addition to the similarity of the natural conditions of these regions, their parks, compared to northern Rivne, also have a significantly higher level of representativeness of dendroflora (Table 2).

To verify the validity of the obtained results in a pairwise comparison of similarity of regional park dendrofloras, the Jacquard similarity coefficient was additionally calculated (the variation was within 0.22 ≤ Kj ≤ 0.30).

Table 2. Matrix of similarity coefficients of regional park dendrofloras of administrative regions

Indicator and the region name of park location		Kj		
		Rivne	Vinnytsia	Zapori-zhzhia
Ksc	Rivne	1	0.22	0.28
	Vinnytsia	0.36	1	0.30
	Zaporizhzhia	0.44	0.47	1

Although much lower quantitative indicators were consequently obtained than when using the Serensen-Chekanovsky similarity coefficient (ΔKj = 0.27), however, the same pattern is generally observed: the highest similarity of dendroflora of the parks of Vinnytsia and Zaporizhzhia, then Rivne and Zaporizhzhia, Rivne and Vinnytsia regions. The reasons for some discrepancies in both cases of application of the similarity coefficients is a significant difference in the number of represented species of woody plants in the regional park dendrofloras of the compared regions. After all, the values of similarity coefficients depend not only on the ratio of the number of common and non-common species of woody plants, but also in direct proportion to the difference between the quantitative numerical values of the species compositions of the compared dendrofloras. However, when using the Serensen-Chekanovsky coefficient, unlike the Jacquard coefficient, the value of such difference is significantly leveled out. At the same time, the Jacquard coefficient requires handling multiple species floras. This feature actually convinced us that it is advisable to use these two similarity coefficients at the same time.

As dendroflora of the parks-monuments of landscape art of Zaporizhzhia and Vinnytsia regions have the highest degree of similarity, it was necessary to consider the common species composition of the parks of the two regions in the taxonomic, biomorphological, ecological and geographical section, as well as separately with the similar list of plant species of Rivne region. As a result of the comparison of the species composition of woody plants in the parks-monuments of landscape art of Vinnytsia and Zaporizhzhia regions, 195 common species were found. They belong to 99 genuses, 38 families and two divisions (*Pinophyta* 15.90% and *Magnoliophyta* 84.10%). *Rosaceae* including 40 species (20.51% of the total) is the best represented among the families. By dividing the total number of species of woody plants by biomorphotypes, we found a significant predominance of mesophanerophytes (48.77%). Common species of plants in this pair of regions represent the following types of life forms: trees (121 species), shrubs (71) and woody vines (3). The height classes are dominated by trees of the first magnitude (53 species) and among the shrubs there is the biggest number of tall plants (32). The analysis of the spectrum of ecological features of common species of woody plants revealed the predominance of heliophytes (54.36%), mesophytes (41.03%), oligotrophs (34.36%) and frost-resistant (64.62%) species. Of the total number of the compared plant species, a significant proportion (68.72%) are plant species exotic for the territory of Ukraine. They originate from the Circumboreal (28 species), Atlantic North American (24), Eastern Asiatic (14), Irano-Turanian (9), Madrean (3), Rocky Mountains (3), and Mediterranean (1) floral regions. The natural habitat of the remaining 46 plant species covers two floral regions at a time, with six species naturally growing within the three floral regions. Instead, most autochthonous species are of the European habitat type and European Nemoral geoelement.

A comparative evaluation of the dendroflora of the parks-monuments of landscape art of Rivne and Zaporizhzhia regions showed that 105 common species of woody plants were found within them, belonging to 62 genuses, 29 families and two divisions (*Pinophyta* 17.14% and *Magnoliophyta* 82.86%). *Rosaceae, Pinaceae and Salicaceae* (17, 11 and 7 species, respectively) were the most representative of the indicated number of families. Six species represent three families, five species only *Cupressaceae*, four species three families, three species four families, two species eight families and one species seven families. According to biomorphotypes 54.29% of 105 species belong to mesophanerophytes. The following types of life forms represent this species composition of woody plants: trees - 73 species (69.52%), shrubs - 29 species (27.62%), woody vines - three species (2.86%). According to the height classes, the major number of species belong to the trees of the first magnitude - 52.05% (*Abies alba* Mill., *Aesculus hippocastanum* L., *Castanea sativa* Mill. etc.) and tall shrubs - 58.62% (*Juniperus communis* L., *Caragana arborescens* Lam., *Cornus alba* L. etc.). According to the analysis of the spectrum of ecological features in the common dendroflora, heliophytyc (54.29%), mesophytyc (40%), frost-resistant (64.76%) and oligotrophyc (37.14%) plant species predominate. Of the total number of common plant species, 41.9% are autochthonous in Ukraine, and 58.1% are dendroexotic plants. Autochthonous plant species are of predominantly European habitat type and European Nemoral and Eastern European Nemoral geoelements. Of the total fraction of dendroexotic plants, only 54.10% naturally grow within one floral region. The largest number of them is the Circumboreal floral region - 15 species of plants. Instead, 23 species represent two floral regions at a time, and four plant species represent three floral regions of the Earth.

Table 3. Taxonomic structure of common species of park dendroflora of Rivne and Vinnytsia regions

Division	Number of families		Number of genuses		Number of species	
	pcs	%	pcs	%	pcs	%
Pino-phyta	3	10.34	8	12.90	21	18.92
Magno-liophyta	26	89.66	54	87.10	90	81.08
Total	29	100	62	100	111	100

As the result of comparison of dendroflora of the parks-monuments of landscape art of Rivne and Vinnytsia regions, 111 common plant species were identified (Table 3). *Rosaceae, Pinaceae*, and *Salicaceae* represent the largest number of plant species (17, 15, and 8, respectively). *Betulaceae* has seven plant species, *Oleaceae* - six, *Cupressaceae* and *Sapindaceae* - five, *Fabaceae*, *Fagaceae* and *Adoxaceae* - four, five families cover three species, seven families - two, the other seven families - one species of plants each. As in the previous version, mesophanerophytyc species of plants predominate by biomorphotype – 54.05%. According to the classification of types of life forms by I.H. Serebryakov (1962) three types have been found: trees (79 species), shrubs (30) and woody vines (2). The height classes in the pair of these two regions are also dominated by species of the first magnitude trees and tall shrubs. As a result of ecological assessment of the composition of common species, the predominance of light-loving plants (54.95%) has been established, with respect to moisture - mesophytyc (38.74%), to temperature - frost-resistant (67.57%), to soil trophicity - the proportion of oligotrophyc and eutrophyc plant species are almost identical (35.14% and 34.23%, respectively). Common species of plants also include dendroautochthons and dendroexotics. Usually a share of autochthonous plant species is 13.52% lower. The European type of habitat and the European Nemoral geoelement are the most well represented by dendroautochthons. Of all dendroexotic plants, 52.38% of plant species are represented by one floral region: Circumboreal - sixteen species, Atlantic North American - eight, Irano-Turanian - four, Eastern Asiatic and Rocky Mountains - two each, Madrean - one plants species.

The native habit of 25 plant species covers two floral regions at once: Circumboreal and Atlantic North American - nine species, Circumboreal and Irano-Turanian - six, Eastern Asiatic and Irano-Turanian - five, two species in pairs represent the Eastern Asiatic and Circumboreal, Mediterranean and Circumboreal floral regions. Four species of plants represent three floral regions: Circumboreal, Atlantic North American and Madrean - two species, Eastern Asiatic, Irano-Turanian and Mediterranean - one species, Rocky Mountains, Circumboreal and Atlantic North American - one species of plants as well.

Finally, as a result of comparison of dendroflora of the parks-monuments of landscape art in Rivne, Vinnytsia and Zaporizhzhia regions, only 89 common plant species were identified, belonging to 56 genuses, 26 families and two divisions. Of these, angiosperms account for a major proportion (80.90%). However, only two families of both divisions dominate by the largest number of plant species (*Rosaceae* and *Pinaceae* - 11 species each). Almost half (48.31%) of the composition of the compared plant species belong to the subtype of mesophanerophytes. Of the three types of life forms, trees (62 species) prevail with slightly fewer shrubs (26). The type of woody vines is represented by only one species (*Parthenocissus quinquefolia* (L.) Planch.). Trees of the first magnitude and tall shrubs have a prominent place by the height classes. Of the eco-groups, heliophytes (53.93%), mesophytes (43.82%), oligotrophs (35.96%) and frost-resistant (61.80%) plants occupy a leading position among the common plant species in the three regions. Among genesis groups of common plant species, there are slightly more dendroexotics (55.06%) than dendroautochthons (44.94%).

Most dendroexotic plants grow naturally within a single floral region: in particular Circumboreal (12 species), Atlantic North American (8), Irano-Turanian (3), Eastern Asiatic (2), Rocky Mountains (2) and Madrean (1) regions. Another 17 plant species cover two floral regions at a time. The rest of the common species grow naturally within the three floral regions. Autochtonous species of woody plants mainly belong to the European type of habitat and the European Nemoral geoelement.

CONCLUSIONS

Despite some differences in the values of both coefficients (Jacquard and Serensen-Chekanovsky indicators), the general trend shows a higher level of similarity of the park dendrofloras, which are found in more similar natural conditions, albeit in different geographical zones (Forest-Steppe and Steppe). In general, most of the common plant species of the studied dendrofloras of the three regions are represented by *Magnoliophyta* and *Rosaceae*. They all belong to a subset of mesophanerophytes as well. A characteristic feature for all three compared pairs of dendroflora is the predominance of trees of the first magnitude, and tall plants among the shrubs. In terms of the range of eco features, most of the compared species are light-loving, mesophytyc and frost-resistant plants. However, there are some differences in the requirements for trophic soils: most common species of park dendrofloras of Rivne and Zaporizhzhia regions have little demand for soil conditions, as they are outside the edaphgeographical optimum of the plant growth and development, regardless of their origin (autochthonous or exotic). On the other hand, oligotrophs and eutrophs are equally common among the common plant species in Rivne and Vinnytsia regions. This difference is due to zonal soil-climatic features. Also, this pair of the compared dendrofloras is characterized by a greater proportion of dendroexotic plants, which indicates a more intensive introductory process in the parks over the historical time of their development. Therefore, an optimal natural-geographical region of Ukraine for park construction and introduction of dendroexotics is the forest-steppe zone. Hence the species of woody plants that are not part of the common species of the pairwise compared dendrofloras shall be introduced in the parks of some natural geographical region.

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ECONOMIC POTENTIAL OF AGRARIAN ENTERPRISES AS AN IMPORTANT DEVELOPMENT FACTOR

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Abstract

The paper aimed to present the evaluation process of the economic potential of agrarian enterprises and its links to the financial results of such enterprises in Ukraine. The study used the data of financial statements of different types of agrarian enterprises of Ukraine and the statistical data provided by the Ministry of Agriculture. The authors proposed updated definitions of "economic potential of the enterprise" and "economic potential of the agricultural enterprise". During the analyzed period, the agricultural holdings had a relatively lower rate of economic potential, than farms. The production of cereals and leguminous has decreased by 1.8%. The meat production's trend line predicted its gradual increase and milk production in 2020-2021 would decline up to 10 million tons if market conditions will stay the same. As a conclusion, the management of the economic potential of agricultural holdings and limited liability companies was more effective, than the one at farms, as bigger producers effectively restored and consistently kept their profitability after crisis reductions.

***Key words**: economic potential, enterprise's potential, agricultural production, agrarian formations, agricultural sector.*

INTRODUCTION

The agricultural production of Ukraine is one of the major sources of state revenues, as well as an important tool for supporting national security by providing its population with high-quality food and other important goods and services.

The agricultural enterprises form the basis of the agrarian sector of Ukraine, where each one of them is a part of a dynamic economic system and its base component. The support and development of all forms of agricultural production require attention from both public and private institutions, including scientific and educational ones.

The economic development of Ukraine has been influenced by the significant internal and external challenges, the consequences of which are market destabilization, weakening the production capacity of different industries, and reducing the purchasing power of consumers.

To maintain the stable market positions of Ukrainian agricultural producers a deep study of agrarian formations and their economic potentials is required.

Studying the economic potential of agricultural formations involves an overview of its theoretical basis, in particular, scientific trends, scientist's findings, previous results, and key definitions.

Existing approaches to the system of economic potential of the enterprise could form three dominant concepts:

- resource concept, according to which the economic potential is a combination of different types of resources;
- functional concept, where the potential is regarded as a set of functions providing of capacities;
- complex concept of resources and targets, according to which the potential is a set of resources and capabilities to achieve concrete results (Prohorova & Bozhanova, 2011).

In the context of the above, the next definitions of the essences of different concepts are given.

Thus, Ansoff I. has expressed the original idea about the nature of the economic potential of the enterprise. He linked the potential of the enterprise with the results of strategic management. The scholar noted that "the potential of the company at the beginning of the production cycle consists of financial, commodity, human resources, and information; at the end of the production cycle it is represented as manufactured profitable products, and services combined with the rules of social behavior enabling an organization to consistently achieve their goals" (Ansoff, 1984).

Kotler F. has expressed a similar idea, explaining the economic potential of the enterprise as the potential of sales that enterprise can do. In addition, he had emphasized that only the demand for the type of products sold by the company may limit the potential of the company sales (Kotler & Zaltman, 1971).

Lepiokhin O. suggested the determination of the economic potential of the enterprise within the same concept. He focused on the idea that the economic potential should be determined as "the aggregate capacity of available economic resources to provide the maximum production of goods and services that are in high demand in the market for profit and meeting social needs". The peculiarity of this researcher's view is that he sees only economic resources as the components of economic potential (Lepiokhin, 2009).

Osipov P. had focused on the study of the links between economic and resource potential and determined the following: "the economic potential is the maximum of resource and production potentials based on the maximum efficiency of elements, forming these resource and production potential" (Osipov, 2004). Using the resources of the enterprise without targeted results in this definition shows that the author also prefers the resource concept.

Summarizing the views of scientists, we offer to use the next definition of the economic potential of the enterprise. It is a complex of resources and capabilities of the enterprise that could be mobilized as needed ensuring the sustainable development of the enterprise through the cooperation of the elements of its potential to provide the market with popular products and services.

The economic potential of an agricultural enterprise is a synthesis of such groups of components as technological and production components, financial and economic components, personnel and social components, biological and ecological components, and unidentified ones. The unidentified components are those have not been included in the named groups of components, but are important for a particular enterprise. Therefore, the economic potential of an agricultural enterprise is the integration of technology and production, financial and economic, personnel and social, biological and ecological, and unidentified components providing maximum profitability of the enterprise within the concept of sustainable development.

MATERIALS AND METHODS

In order to characterize the economic potential of agrarian enterprises of Ukraine production of cereals, leguminous, milk, meat, and activities of 8 agricultural producers were studied. Calculations of the economic potential of an enterprise required the collection of statistical data on its components. To assess it we have formed a system of indicators in accordance with grouping principles of economic potential components, in particular, the technological and production component of economic potential can be represented by such indicators (Taraniuk, 2014) (Table 1).

Table 1. Indicators and symbols of technological and production components of the economic potential of agricultural enterprises

Indicators	2016	2017	2018
Produce	P_1	P_2	P_3
Work-in-progress	W_1	W_2	W_3
Goods and services	G_1	G_2	G_3
Inventories	I_1	I_2	I_3
Accounts receivable	AR_1	AR_2	AR_3
Accounts payable	AP_1	AP_2	AP_3

Source: proposed by authors

Next step is to transform the collected data into a homogeneous group. To do it we have compared the data with the benchmark indicator, which is the maximum indicator of each row (1).

$$Yi = \frac{Xi}{Xei} \quad (1),$$

Y*i* - adjusted indicator;

X*i* - indicator of row *i*;

Xe*i* - benchmark of row *i*, Xe*i*=1.

Table 2 presents the procedures of transformation of technological and production components of economic potential.

Table 2. Transformation of technological and production components of economic potential

Indicators	2016	2017	2018
Produce	P_1/P_3	P_2/P_3	1
Work-in-progress	W_1/W_2	1	W_3/W_2
Goods and services	1	G_2/G_1	G_3/G_1
Inventories	I_1/I_2	1	I_3/I_2
Accounts receivable	AR_1/AR_3	AR_2/AR_3	1
Accounts payable	1	AP_2/AP_1	AP_3/AP_1

Source: compiled by authors

The suggested approach helps to calculate the integrated indicator of each component and the aggregated economic potential indicator of studied agricultural enterprises.

The next step of the calculation process is the calculation of the index of each component by using the formula below. As an example, the index of technological and production components in 2017 (2):

$$TP_{2017} = \sqrt{\prod_{i=1}^{m} Y2017} \quad (2)$$

After the calculation of components' indexes, we have to summarize the indicator of the economic potential of the enterprise. The components of economic potential could have different weights, and therefore it is needed to determine the coefficient for every component. To achieve it, the focus group of experts of agribusiness and economists has evaluated the significance of every component for such analysis. The results of their evaluation are in Table 3.

Table 3. Coefficients for the economic potential components of agricultural enterprises based on expert opinions (example)

The components of the economic potential	Points	Coefficient (*k*)
Technology and production	35	0.35
Personnel and social	20	0.2
Financial and economic	22	0.22
Biological and ecological	18	0.18
Unidentified	5	0.05
Total	100	1

Source: compiled by authors

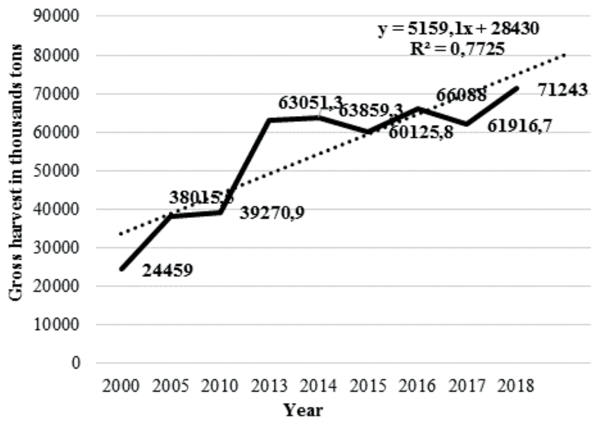
After the determining the coefficients and component values of economic potential, a basic formula for the integrated indicator of economic potential could be designed (3):

$$\boldsymbol{E_{pl}} = k_{TP} \times TP + k_{PS} \times PS + k_{FE} \times FE + k_{BE} \times BE + k_{U} \times U \quad (3)$$

TP, PS, FE, BE, U - component values of economic potential;
k_{TP}, k_{PS}, k_{FE}, k_{BE}, k_{U} - coefficients of components of economic potential.

RESULTS AND DISCUSSIONS

To evaluate the dynamics of cereals and legumes cultivation in Ukraine we have designed a trend line based on the statistical data of 2000-2018 (Figure 1).



Source: created by authors using (Prokopenko, 2018)

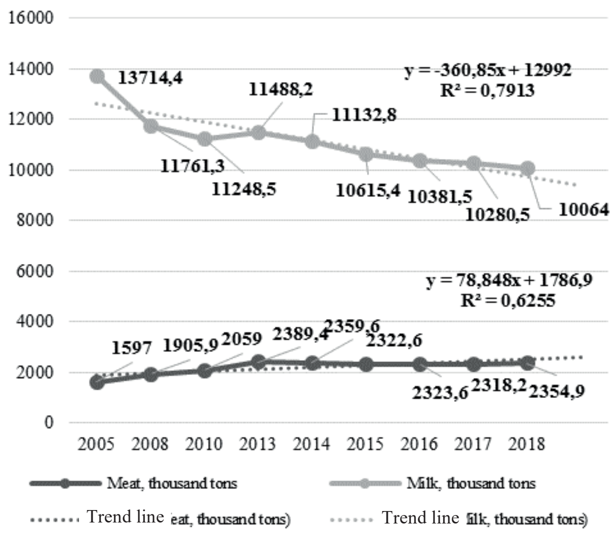
Figure 1. The graph of the dynamics of cereals and leguminous cultivation in Ukraine in 2000-2018, thousands of tons

During the study period, the production of cereals and leguminous has decreased by 1.8%. A significant decline in the production of these crops had been recorded in 2015 and 2017. The reduced production volumes could be explained by a set of reasons, including changes in the structure of production of such crops; the implementation of reforms directly related to land resources and methods of their governance (decentralization); such critical climatic issues, as extremely high summer temperatures and periods of heavy rainfall. According to the trend line of the cereals and leguminous production, their gross collection with high probability (determination coefficient is over 0.77) will continue to increase and in 2020-2021 could reach 80 million tons.

In 2018, the production of these crops after a slight fluctuation in 2015-2017 exceeded the level of 2014. When comparing the data of 2018 with data from 2000 it appears that the current production level is almost three times higher than in 2000.

Statistics show that during the study period the level of meat production has hardly changed and at the beginning of 2017 amounted to 2318.2 thousand tons, which is 3% less than in 2013.

To evaluate the prospects for the economic development of agricultural meat and dairy producers we have conducted a trend analysis based on the statistics of raw meat and milk production during 2005-2018 (Figure 2).



Source: created by authors based on (Prokopenko, 2018)

Figure 2. The graph of the dynamics of raw meat and milk production in Ukraine, 2005-2018

The equation of the trend line of milk production has a negative coefficient of argument X (determination coefficient is 0.79), describing a gradual decline in milk production. The resulting trend line shows that milk production in 2020-2021 will decline up to 10 million tons if market conditions will stay the same. Reducing milk production will lead to a new wave of appreciation for dairy products because the demand for such products is consistently high.

The meat production's trend line demonstrates and predicts its gradual increase. In a few subsequent years, meat production can amounted to 25 million tons. Studying the meat production indicator in 1990 (almost 43 million tons), it appears that the projected production level will reach only 58% of it (Prokopenko, 2018).

To study the economic potential 8 agricultural enterprises were selected: 4 agrarian holdings, 2 limited liability companies, and 2 farms.

The assessment of the economic potential of selected agrarian enterprises has been held in accordance with the calculation approach proposed. The coefficient of each component of the economic potential has been determined using a survey of ten experts. The results of survey are in Table 4.

Table 4. The coefficients of basic components of economic potential

The components of the economic potential	Coefficient (*k*)
Technology and production	0.3
Biological and ecological	0.35
Financial and economic	0.15
Personnel and social	0.2

Source: calculated by authors based on experts' survey results

At the end of the calculation process, the following results appeared (tables 5-8). To compare the dynamics of calculation results and financial results of studied agricultural enterprises during 2014-2018, the EBITDA index has been added to the tables.

During the study period, agricultural holding "Avangard" gradually lost the pace of development. Thus, in 2014 the EBIDTA of this holding amounted to 98.2 mln. USD. The next year it decreased by 9 times and in 2018 it had a negative value.

Having considered the index of economic potential, it has been concluded that during the period of EBITDA declined in 2015-2017 the holding has started to use its potential in a not optimal way. This conclusion could be confirmed by the fact that during the period of the greatest decline in EBITDA, the economic potential has also halved and remained at this level until 2018. At the end of the study period, the holding optimized its economic potential to a higher level comparing to its indicator at the beginning of the crisis (Table 5).

Table 5. The economic potential and financial results of the agrarian holdings "Avangard" and "Kernel", 2014-2018

Indicators	Years									
	2014	2015	2016	2017	2018	2014	2015	2016	2017	2018
	Agrarian holding "Avangard"					Agrarian holding "Kernel"				
EBIDTA, mln. USD	98.2	12.6	1.5	11.8	-0.9	223	396.6	346.4	319.2	222.5
Economic potential of agrarian holdings	0.31	0.18	0.19	0.18	0.38	0.48	0.34	0.23	0.35	0.48

Source: calculated by authors using (Avangardco, 2014, 2016, 2018; Kernel Holdings, 2014, 2016, 2018).

During 2014-2018, agrarian holding "Kernel" demonstrated consistently high financial results with a decrease at the beginning and at the end of the study period. The dynamics of the economic potential of this holding show that during the reduction period of EBITDA the management has promptly liquidated negative phenomena hindering further development (Table 6).

Table 6. The economic potential and financial results of the agrarian holding "Astarta" and "MHP", 2014-2018

Indicators	Years									
	2014	2015	2016	2017	2018	2014	2015	2016	2017	2018
	Agrarian holding "Astarta"					Agrarian holding "MHP"				
EBIDTA, mln. USD	104.3	114.1	132.9	105.0	49.7	555	459	415	367	311
Economic potential of agrarian holdings	0.13	0.19	0.42	0.49	0.42	0.34	0.35	0.43	0.53	0.76

Source: calculated by authors using (Astarta Holding, 2016, 2018; MHP S.A., 2014, 2016, 2018)

During the study period, agricultural holding "Astarta" received a high value of EBITDA in 2016, but it has fallen almost threefold by 2018. Having considered the indicators of economic potential, it can be concluded that the managers have changed the strategy of the holding's development in 2015-2016, as the figures have doubled and remained at that high level until 2018. The sharp decrease of EBITDA is explained by highly competitive market conditions or other complications that have not allowed implementing the potential generated previously (Astarta Holding N.V., 2016, 2018; MHP S.A., 2014, 2016, 2018).

During 2014-2018, the "MHP" holding gradually lost a high level of profitability, at the same time, the company was increasing its economic potential in order to level the situation and regain the high income as in previous periods. During 2016-2018, the "MHP"'s economic potential grew by 46%, but there was no corresponding revenue growth. Such situation is explained in the same way as in the case of "Astarta", the market failed to respond accordingly to the strategic steps of "MHP", and external factors have limited the desired profitability of these agricultural holdings (Astarta Holding N.V., 2016, 2018; MHP S.A., 2014, 2016, 2018).

The agricultural enterprises that are limited liability companies were analyzed according to the same approach (Table 7). Two companies from the Khmelnyts'ky region engaged in crop and animal husbandry were selected for this analysis.

Table 7. The economic potential and financial results of the agrarian enterprises "Podillia+", LLC and "Lotivka Elit", LLC, 2014-2018

Indicators	Years									
	2014	2015	2016	2017	2018	2014	2015	2016	2017	2018
	"Podillia+", LLC					"Lotivka Elit", LLC				
Financial result, thousand USD*	55.70	95.52	142.27	177.24	85.22	1149.83	1698.99	2057.98	6033.19	36.06
Economic potential	0.15	0.21	0.21	0.54	0.41	0.41	0.43	0.45	0.60	0.51

** exch. rate 23.68 UAH per 1 USD as of 01.01.2020*

Source: calculated by authors using financial statements of the farms

The analysis has shown that "Podillia +", LLC at the beginning of the study period, as the previously studied holdings, was able to achieve high revenues, and by its end, the company has been experienced a significant revenue decline. In 2017, the company has increased its economic potential, which has increased financial results respectively. However, in 2018, there has been a decline in it. Given the growing economic potential, it can be assumed that the next two years the enterprise will be able to withstand the challenges and increase the income.

"Lotivka Elit", LLC has demonstrated the similar dynamics of profitability in 2014-2018. In 2018, the financial result of the company has fallen by almost a hundred times over the previous year, which also led to a decline in economic potential. Therefore, the company has had resources to restore the optimal level of profitability in the years that followed.

Next, we have analyzed the indicators of financial results and economic potentials of selected farms according to the same algorithm (Table 8).

Table 8. The economic potential and financial results of the farms "Molochars'ke-M" and "Liubymivs'ke", 2014-2018

Indicators	Years									
	2014	2015	2016	2017	2018	2014	2015	2016	2017	2018
	"Molochars'ke-M" farm					"Liubymivs'ke" farm				
Financial result, thousand USD*	8.36	8.53	4.56	13.51	14.57	4.69	6.50	2.62	5.07	2.41
Economic potential	0.80	0.65	0.63	0.67	0.82	0.49	0.58	0.54	0.76	0.72

**exch. rate 23.68 UAH per 1 USD as of 01.01.2020*
Source: calculated by authors using financial statements of the farms

During the study period, "Molochars'ke-M" farm, just like other studied enterprises, have had fluctuations in the financial result. Nevertheless, unlike the others, at the end of that period, this farm has managed to increase its financial results. It should be noted, that its level of economic potential was larger than 0.5 throughout the study period meaning a high level of farm resources consolidation. In addition, the farm has been able to optimize its market strategy leading to an increase in the net financial results.

Two times the "Liubymivs'ke" farm had been receiving the annual financial result lower than USD 3k during 5 years of the study period, although the level of its economic potential in both cases has exceeded 0.5. Therefore, the farm's poor product quality, improper logistics or marketing strategy could be problematic for its development.

CONCLUSIONS

As a result of the study, the authors proposed the definitions of the economic potential of enterprises and agrarian formations. Thus, the economic potential of enterprise is a complex of resources and capabilities of the enterprise that could be mobilized as needed ensuring the sustainable development of the enterprise through the cooperation of the elements of its potential to provide the market with popular products and services.

The economic potential of an agricultural enterprise is the integration of technology and production, financial and economic, personnel and social, biological and ecological, and unidentified components providing maximum profitability of the enterprise within the concept of sustainable development.

Analysis of the performance of agricultural producers using the proposed calculation methodology of the economic potential helped to assess the condition and dynamics of resource use by the companies, their potential opportunities, and strategies of their implementation. Thus, agricultural holdings have a relatively lower rate of economic potential, than farms, which indicates a higher level of consolidation of farm resources. However, the management of the economic potential of agricultural holdings and limited liability companies was more effective, than the one in farms, as bigger producers effectively restored and consistently kept their profitability after crisis reductions. The general trend of economic potential indicators was its significant decrease during the 2014-2015 crisis and in 2018.

The received calculations help to assess the tendencies of agricultural production and the economic potential of agricultural enterprises. The achieved results are the basis of forecasting the results of the agricultural enterprises' activities in the coming years.

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CONSIDERATIONS REGARDING THE USE OF EXPERIMENTAL ANIMAL MODELS IN DENTAL MEDICINE - A LITERATURE REVIEW

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Abstract

The purpose of the study is to document in the specialized literature, the importance of using experimental animal models in dental medicine. For this study we reviewed 41 abstracts of scientific papers and 81 in-extenso articles, using Web of Science Core Collection and PubMed databases. Additionally, a search in 5 specialized books was performed. Of these scholarly papers, 67 were considered to be relevant for this study. Even though the prevalence of these studies is decreasing due to the new legislative restrictions, the scientific results obtained with the use of these models in medical research are evident. The most relevant experimental animal model for scientific documentation of oral rehabilitation is the dog, both for dental implant surgery research and for the study of biomaterials used in the treatment of periodontal disease. The monkey is considered to be of excellent relevance for the evaluation of healing after periodontal treatments.

Key words: *experimental animal model, oral rehabilitation, dental implant*

INTRODUCTION

Experimental research involves choosing an experimental model appropriate to a certain predetermined scientific purpose.

The use of animals for experimental purposes is a fairly old scientific practice, which is still under development due to its advantages. For example, in dental medicine, many studies were performed using experimental models: research regarding dental implants osseointegration, epithelium and connective tissue attachment to dental implants, integration of bone grafts or studies for evaluation of pulpal inflammation.

The number of animals used in experiments remains high even in the third millennium. In Germany, for example, in 2001, approximately 2,126,000 animals were used in experiments (Sălăvăstru, 2014).

Similarities regarding embryological development and morphological resemblances in animals and humans are the basis of experimental studies.

Experimental animal models were recommended after performing histological and immunohistochemical studies that showed their possible clinical efficacy. In this regard, fragments of different tissues were collected from the experimental specimens, and then the pieces containing the areas of interest were analyzed immunohistochemically and histologically. Samples processing for the immunohistochemical and histological analysis must be in accordance with the legislation in force and with the recommendations of the medical practice guidelines for Anatomical pathology (Poll, 2015).

Also, the development of experimental models must be in accordance with the provisions of the Council of Europe Directive no. 86/609/ EEC on the protection of animals used for experimental or other purposes, with the Fifth Report on the statistics on the number of animals used for experimental purposes in EU Member States, ECA SEC 1455, Brussels (2007), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and with the regulations in force and with other regulations.

With the help of scientific literature data, the study presents a series of observations regarding the role of experimental animal models in scientific documentation of oral rehabilitation.

For experimental research in dental medicine, mainly for research related to periodontics and implant dentistry, it is recommended to use larger animal models and adult animals with a slower growth rate. Bones similar to the human maxilla and mandible, in what concerns the ratio of compact to spongy components, are preferred for the study of dental implant osseointegration (Babuska, 2016).

MATERIALS AND METHODS

A systematic search in the scholarly literature was conducted, in order to identify studies on the use of experimental animal models in scientific research in the field of dental medicine. 41 abstracts of scientific works and 81 in-extenso articles were accessed using Web of Science Core Collection and PubMed databases and the following key words: experimental animal model in dental medicine. Additionally, a manual search in five specialized books was performed. Of these scholarly papers, 67 were considered to be relevant for this study.

For the reasons presented above, this study analyzed in particular bibliographic references relevant for the use of experimental canine models.

RESULTS AND DISCUSSIONS

An animal model is defined as a non-human living animal with an inherited naturally acquired or induced pathological process or lesion allowing for the resolution of a research hypothesis and resembling a similar condition in the target human species (Hau et al., 1989).

In dental research, the animal models have particular applications for the study of periodontal disease and dental implant treatment. Initially, the pathogenesis of oral diseases, including experimental periodontitis, was aimed to be explored (Staubli et al., 2019). In recent years, animal experiments have been applied with respect to the performance of dental implants or the pathogenesis and therapy of peri-implant diseases (Staubli et al., 2019; Ericsson et al., 1996).

Small animals were rarely used as experimental models in dental medicine because of their oral, maxillary and mandibular anatomy which is different from the one of the human species. Consequently, small animal models have reduced clinical utility for dental medicine.

In the accessed references, we did not find examples of studies on **mice** that analyzed the dental implant integration at the level of the maxilla and mandible, the field of interest in this regard being not the intraoral bones, but the bones of the lower limb, such as the femur.

Because **the rat** jaws and teeth are small, the rat is mainly recruited for research on dental implants placed into the calvaria, the tibia, or the femur (Dard, 2012). The author reveals that only few studies dealt with intra-oral placed implants, and they described mini-implants placement into jaw bones, rather than implants placed for teeth replacement (Dard, 2012). Trabecular bone growth was examined around titanium mini-implants placed in rat femurs (Shimizu-Ishiura et al., 2002). Haga and coworkers are the ones who documented the bone formation and maturation around dental implants placed in the rat maxilla (Haga et al., 2009).

The rat is the most extensively studied rodent for the pathogenesis of periodontal diseases. A considerable difference exists between the human species and the rat, in that the rat is extremely resistant to periodontal disease (Struillou et al., 2010).

The gilded hamster (*Mesocricetus auratus*) remains the most interesting model for immunological research (Struillou et al., 2010).

A more systematic use of small animal models (rat and hamster) is recommended for future research on surgical treatment of periodontal disease (Struillou et al., 2010).

We have found only two publications that described studies in which **the cat** was used as an experimental animal for dental medicine research purposes (Takahashi et al., 2005; Silva et al., 2012).

The rabbit, which represents the animal of choice in about one-third of all musculoskeletal studies (Neyt et al., 1998), is also commonly used in implant dentistry (Dard, 2012). The rabbit was used as 'pre-translational animal model' in implant dentistry, for assessing dental implant designs and materials prior to testing them in a larger animal (Dard, 2012).

The rabbit has mainly been used for testing biomaterials or for investigating the treatment of peri-implantitis (Struillou et al., 2010).

In comparison to other species, such as primates, the rabbit has a faster bone turnover with significant intracortical, haversian remodeling (Pearce et al., 2007; Dard, 2012; Mapara et al., 2012).
Bone healing after guided tissue regeneration (well-known method in periodontal surgery), was studied on rabbit tibia model (Aaboe et all., 1994). Bone healing after application of PerioGlas (silicate-based synthetic bone augmentation material) in surgically created defects adjacent to titanium plasma-sprayed dental implants was studied on rabbit tibia model (Johnson et al., 1997). Bone regeneration promoted by porous bone mineral and biologically active glass (materials used for achieving alveolar bone augmentation and periodontal regeneration) was assessed on rabbit radius model (Schmitt et al., 1997). Although long bones, like tibia and radius represent interesting models for investigating the bone healing, they can not replicate the anatomical and physiological particularities of the maxilla, the mandible and of the alveolar bone (Struillou et al., 2010).
The accessed publications showed us that the number of experimental models that used sheep or goat for assessing the integration of dental implants is increasing, due to the dimensions of the maxillary and the mandibular bones in these animals, which are similar to those in the human species. However, there are no recent publications available using sheep as experimental animal model for research in periodontics (Struillou et al., 2010).
The swine is one of the most used animal species for translational purposes in pharmaceutical research. Over the last two decades, it has been positioned as a candidate among other species for use in musculo-skeletal surgical investigations (Dard, 2012).
The swine has bone remodeling processes similar to humans, comprising both a dense trabecular network and intra-cortical remodeling and it shows similarities in bone mineral density and bone mineral concentration to human bones (Mosekilde et al., 1987; Mosekilde et al., 1993; Aerssens et al., 1998). The miniature pig (micro- or minipig) offers several advantages over the domestic swine for dental implant research purposes. The advantages are mainly related to handling, housing and administering anaesthesia (Dard, 2012). The size, shape and anatomy of the minipig mandible and the movement of the temporomandibular joint in minipig are similar to those of humans (Dard, 2012).
Monkeys have the advantage of being philogenetically similar to humans (Struillou et al., 2010). The structure of the periodontium is also histologically similar to that observed in humans (Struillou et al., 2010). The inflammatory response to periodontal disease is quite similar to that found in humans: connective tissues are infiltrated by plasma cells, lymphocytes and neutrophils (Struillou et al., 2010). In some species, such as squirrel monkeys and marmosets, there is very limited inflammatory infiltrate. This major difference from humans makes them inappropriate models for studying the pathogenesis of periodontitis (Page and Schroeder, 1982; Struillou et al., 2010). The literature describes the monkey as an adequate experimental animal model for implant dentistry research, because of its oral healing characteristics similar to those of humans (Dard, 2012). However, clear ethical considerations limit the use of this animal model for surgical research purpose (Dard, 2012).
Monkey experimental models have been used for periodontal healing and biomaterials investigations (Drury et al., 1991; Ling et al., 1994; Sculean et al., 1997; Karatzas et al., 1999; Sculean et al., 2000) and evaluation of bone remodeling around loaded dental implants (Piattelli et al., 1998; Scarano et al., 2000; Piattelli, et al., 2003). Macaca fascicularis is the most used species for research on periodontal and dental implant surgery.
As *Macaca fascicularis* has the same dental formula as human, all the teeth can be used, which makes it possible to obtain an important number of test sites, with a limited number of animals (Struillou et al., 2010).
First introduced in the 1960s for research in periodontics, **the dog** has remained popular as model for studies on periodontal surgery. In this field, the studies address both spontaneous and experimental periodontitis in order to understand the etiopathology of the disease, its semiology, and the mechanisms of periodontal destruction and healing (Hennet, 1999).

Based on history and its previously broad use, one of the most preferred animals for research related to implant dentistry is *the Beagle dog* (Dard, 2012). However, this animal is highly affected by spontaneous periodontitis (Kortegaard et al., 2008; Dard, 2012).

Experimental animal models are useful for evaluating the behavior of biomaterials and the success of clinical procedures, as *in vitro* models cannot replicate the complexity of the human anatomy. It would be ideal that an experimental model for the study of the integration of bone autografts to have, embryologically and morphologically, similar biological traits in the donor and recipient areas (Poll et al., 2018; Isaksson, 1992; Klinge et al. 1992; Poll et al., 2018).

Research in dentistry has described different experimental animal models, such as the experimental model using rabbit as specimen (Atiya et al., 2014), the experimental model using rat as specimen (Korn et al., 2014; Levy et al., 2013), the experimental model using goat as specimen (Zou et al., 2012), the experimental model using pig as specimen (Ogunsalu et al., 2011), the experimental model using cat as specimen (Silva et al., 2012).

Other authors used the dog as model for experimental research in order to study bone regeneration in the case of autografts applied at the maxillary level (Pourebrahim et al., 2013; Oryan et al., 2014; Poll et al., 2018; Nimigean et al., 2019).

In the last decade many experimental studies on animal models were developed in order to increase the long-term performances of dental implants. These studies have shown that larger segments of bone autografts ensure a better conservation of bone volume and maintain bone height eight weeks after being applied (Ogunsalu et al., 2012; Ogunsalu et al., 2013; Kon et al., 2014).

Bone healing is a complex biological phenomenon that takes place both during the growth of the body and during its development stages, as well as in certain bone modeling, remodeling and repair processes. The necessary conditions for healing after surgery are mainly represented by: adequate blood supply, lack of connective tissue at the interface, and primary stability of the grafts (Poll, 2015).

On such experimental models, the integration of dental implants and autogenous bone grafts can be further studied through histopathological and immunohistochemical investigations, like are studied, for example, on dental pulp models the histological and immunohistochemical changes of pulp tissue exposed to different biomaterials (Nimigean et al., 2016; Nimigean et al., 2018; Nimigean et al., 2019; Tuculina et al., 2013).

It can be stated that the immunohistochemical assessment of the integration of mandibular autografts applied in maxillary bone defects, represents a valuable technique for the evaluation of initial and early phases of healing, statement similar to the conclusions of other authors (Schwarz et al., 2007). However, for the histological evaluation to be relevant, an increased resolution is required in order to distinguish the qualitative differences of the tissues, as other authors also have shown (Friedmann et al., 2014).

Immunohistochemical investigations regarding the relevance of experimental animal models have been less mentioned in the medical literature. As other studies showed, immunohistochemical investigations are rare or absent even in clinical research on certain infrequent pathological conditions (Vija et al., 2014).

The integration of the bone graft in the receiving area also depends heavily on its adequate revascularization, as it is independent of the vascular support of the receiving area (Elsalanty and Genecov, 2009).

The proliferation of bone cells is responsible for tissue regeneration, and osteocyte survival in the grafted areas depends directly on the blood supply and on the vitality of the periosteum (Salgado et al., 2011).

Particular morphologic patterns both at vascular level and at bone level (such as the relation of the mandible to the maxillae) might suggest predisposition to certain disorders and complications (Enache et al., 2010; Nimigean et al., 2018).

The experimental animal models are relevant for the study of periodontal disease, dental implants, biomaterials and new regenerative strategies in dental medicine (Struillou et al., 2010).

The relevannce of different animal models for research on periodontal treatment and dental implant surgery is presented in the Table 1 (Struillou et al., 2010).

Table 1. The relevance of different animal models for research on periodontal treatment and dental implant surgery (Struillou et al., 2010)

Species	Relevance according to research topic	
	Biomaterials for periodontal treatment	Dental implant surgery
Non-Human Primates	Excellent	Good
Dog	Excellent	Excellent
Minipig	Good	Medium
Rabbit	Medium	Medium
Rat	Medium	Low
Hamster	Low	Low

Experimental animal models are essential to understanding the origin and evolution of the periodontal diseases pathology in humans. The most commonly used animal model in periodontal research is the dog, due to the reproducible critical-sized defects. Many experimental studies on gingivitis and periodontitis have been conducted in dogs. The Beagle is one of the most commonly used due to its size and its extremely cooperative temperament (Struillou et al., 2010).

Studies in dogs have also reported research on mesenchymal stem cells and tissue engineering in the treatment of periodontal disease (Struillou et al., 2010).

The only criterion for the choice of a certain experimental research topic is its relevance, which cannot, however be judged in the short-term. A concept to systematically assess the relevance of the in vitro tests must be developed in order to increase quality and to finally achieve an evidence-based biomedical research (Gruber and Hartung, 2004).

An important role is attributed to the experimental salivary models, which do not have legislative restrictions, for example, Stefanescu et al. 2011, “Salivary monitoring of hormone levels has many advantages over the more conventional serum/plasma analysis”.

The use of experimental animal models in scientific research favors obtaining relevant and useful information for the human species.

However, experimental models are limited by scientific constraints and due to increased regulatory constraints (Knight, 2008; Leist et al., 2008; Lilienblum et al., 2008).

Experimental animal models have been used in dental and peri-implant research and have been a subject of debate in recent years (Staubli et al., 2019). Critical remarks have been published with respect to the transposing of research data derived from animals to the pathogenesis and therapy of human diseases and with respect to the ethical considerations on using experimental animal models for biomedical research (Staubli et al., 2019).

Like any chronic disease, periodontitis is a multifactorial pathological condition. The onset and progression of the periodontal disease are caused by the infection with a pathogenic oral biofilm. The composition of the oral biofilm is highly variable, showing intra- and interindividual diversity and it is affected by several behavioral factors, like nutrition and tobacco use and by local environmental factors, like the quality, the extent and the material of dental prostheses (Staubli et al., 2019). Moreover, scientific evidence indicates education and lower socio-economic status as risk factors for periodontal disease (Rodriguez et al., 2017). It is difficult, even impossible, to simulate most of the variables listed above in experimental animal models. Therefore, it is appreciated that animal experiments do not provide direct evidence relevant for human periodontal or peri-implant diseases (Staubli et al., 2019). The benefit for understanding pathogenesis or directions for therapy of human diseases needs to be critically defined for each experiment and for each animal used (Staubli et al., 2019).

Furthermore, a high standard of analysis and reporting of data obtained from animal research and adherence to quality guidelines such as the ARRIVE (animal research: reporting in vivo experiments) guideline are required to reduce the risk of bias derived from animal experiments (Kilkenny et al., 2010; Staubli et al., 2019).

A reduction in the number of experimental studies on animals for dental medicine research purposes has been registered during the last decade. This is in accordance with the implementation of the 3Rs principle - replacement, reduction, and refinement of the use of animal models in biomedical research (Pasupuleti et al., 2016).

CONCLUSIONS

The experimental animal models are useful for understanding the oral pathology in humans.

The literature review is beneficial in evaluating the relevance of experimental animal models for dental medicine research purposes, because the different indications regarding their use require tailored solutions.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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INTERDEPENDENCE RELATIONSHIPS BETWEEN AGROCHEMICAL INDICES FOR CHARACTERIZATION AN AGRICULTURAL LAND

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Abstract

This study evaluated the relationship of interdependence between agrochemical indices of the soil in the pedoclimatic conditions of Beregsău area, Timiș County, Romania. The agrochemical indices that characterize the agricultural soil, were taken into consideration: soil pH, nitric nitrogen (NO_3^-), ammoniacal nitrogen (NH_4^+), mineral nitrogen (Nmin), phosphorus (P_2O_5), potassium (K_2O), secondary macro-elements (Ca, Mg, S), sodium (Na), and microelements (Fe, Mn, Cu, Zn, and B). Very high positive correlation was recorded between Nmin and NO_3^- (r = 0.990), and very high negative correlation was recorded between Mn and pH (r = -0.973). High positive correlations were recorded between Cu and K (r = 0.857), between Cu and Mg (r = 0.834), and between Na and Mg, respectively (r = 0.893); high negative correlation was recorded between Mg and Ca (r = -0.855). Moderate positive correlations were recorded between B and pH (r = 0.783), between Mg and K (r = 0.700), and moderate negative correlations were recorded between Ca and K (r = -0.738), and respectively between Na and Ca (r = -0.703). Based on the values of the coefficients of variation (CV) it was appreciated that pH, and potassium had the highest degree of uniformity in the characterization of the studied soil. High degree of variation was recorded in the case of nitrogen (NO_3^-,NH_4^+, Nmin), phosphorus, followed by secondary macro-elements, and micro-elements. The interdependence relationships between certain agrochemical indices were described by different mathematical model in the form of linear equations (NO_3^- and Nmin, in condition of R^2 = 0.981, p << 0.001; Mn and pH, in condition of R^2= 0.947, p << 0.001), and in the form of polynomial equations, respectively (Mg and Ca, in condition of R^2 = 0.779, p = 0.0107; Cu and K, in condition of R^2 = 0.768, p = 0.012; Cu and Mg, in condition of R^2 = 0.819, p = 0.0059). PCA explained 98.7808% of variance in relation to the main soil macro-elements, 99.9986% of variance in relation to the secondary soil macro-elements and 99.8228% of variance in relation to the soil micro-elements, respectively.

Key words: *agrochemical indices, correlations, mathematical model, PCA, soil.*

INTRODUCTION

Agricultural soil and land fertility is periodically evaluated on the basis of general quality indices and specific agrochemical indices that define soil reaction, soil organic matter, supply with main and secondary macroelements (N, P, K, S, Ca, Mg), microelements (Fe, Mg, Cu, Zn, B, Mo), cation exchange capacity (CEC), saturation degree in basic cations, electrical conductivity (EC) etc. (Plaster, 2003; Havlin et al., 2005; Jónsson et al., 2016).

Knowing these agrochemical indices is important for establishing soil health and improvement measures, evaluating the relationship between plants and soil, managing fertilization plans and optimizing fertilizer doses (Idowu et al., 2009; Gelaw et al., 2015; Boldea et al., 2015; Andriucă et al., 2018; Cojocaru and Cerbari, 2018; Norris and Congreves, 2018; van Es and Karlen, 2019).

Soil quality indices and soil supply level with mineral nutrients, varies in time and space in relation to the type of soil, agricultural technologies level, crops structure and rotation, yields, farm management, natural and anthropogenic factors of influence (Santillano-Cázares et al., 2012; Wang et al., 2014; Congreves et al., 2015; Apesteguía et al., 2017; Roper et al., 2017; Su et al., 2018).

Carbon fractions and changes of soil organic carbon, were evaluated according to diversity and crop rotation, soil tillage systems, pedoclimatic conditions (Weil et al., 2003; Alhameid et al., 2017; Bongiorno et al., 2019).

Soil pH has been intensively studied in relation to soil type, microbiological activity, precipitation regime, type and dose of ameliorative substances and fertilizers used, agricultural crops, watering system, soil tillage system, etc. (Bolan et al., 2003; Aciego Petri and Brookes, 2008; Goulding, 2016; Ghimire et al., 2017; Bai et al., 2018).

Soil fertility and macroelement regime were studied in relation to soil type and its characteristics, climatic conditions, land use, crops and yields, agricultural practices, fertilizer inputs, nutritional imbalances in plants (Rolando et al., 2018; Cojocaru, 2019; Datcu et al., 2019; Sala et al., 2019; Willy et al., 2019).

Soil microelements regime was studied in relation to soil type, soil pH, soil organic matter, macroelement content, especially those with antagonistic effect (eg. Ca^{2+}), cultivated plants, fertilizers application methods and techniques, production quality (Jivan and Sala, 2014; Zhao et al., 2014; Rawashdeh and Sala 2015a;2015b; 2016; Dhaliwal et al., 2019; Ma et al., 2019).

The spatio-temporal variation of soil fertility and influence on agricultural crops has been studied both by classical, analytical or quantitative method (Andrews et al., 2004; Moebius et al., 2007; Kinoshita et al., 2017; Rinot et al., 2019), and by imagistic methods which are increasingly promoted as a result of the advantages presented, especially for precision agriculture (Yasrebi et al., 2008; Li et al., 2012; Herbei and Sala, 2015; 2016; Vohland et al., 2017; Song et al., 2018).

Some agrochemical indices have greater stability in soil fertility characterizing, while others have much more dynamic variation. Soil organic matter content has a high stability (Kirkby et al., 2011; Clercq et al., 2015), while nitric and ammoniacal nitrogen have an increased dynamics (Nurulhuda et al., 2018; Pacifico et al., 2019).

Soil pH varies with soil type and agricultural practices, in particular with the type and doses of fertilizers used, with crop structure, with watering regime, etc., and in turn the pH influences the nutrients regime in soil (Jia et al., 2009; Marinca et al., 2009; Vašák et al., 2015).

Between agrochemical indices of the soil there is a close interdependence (Marschner, 1995; Havlin et al., 2005).

Present study evaluated the interdependence relationships between agrochemical indices that define the fertility of the soil, in field crops conditions.

MATERIAL AND METHODS

The study evaluated the fertility status and the relationships of interdependence between the agrochemical indices of the soil, chernozem type, in the pedoclimatic conditions of Beregsău area, Timiș County, Romania.

Specific agrochemical indices, currently used for the characterization of agricultural land, have been studied. Soil samples were taken at 0-30 cm depth from plot BF 506, and agrochemical indices were determined: soil pH, nitric nitrogen (NO_3^-), ammoniacal nitrogen (NH_4^+), mineral nitrogen (Nmin), phosphorus (P_2O_5), potassium (K_2O), secondary macro-elements (Ca, Mg, S), sodium (Na), and microelements (Fe, Mn, Cu, Zn, B). The soil sample analysis was performed by SC Vantage Balkans SRL (2018), using accredited laboratory methods and software.

The statistical analysis of the experimental data was done with the statistical calculation module from EXCEL, Office 2007 and with the PAST software (Hammer et al., 2001).

Single-factor ANOVA test, correlation analysis, regression analysis, coefficient of variation (CV), and PCA were performed. For the statistical certainty of the results, the correlation coefficients r and R^2, the parameter p, and F test were used.

RESULTS AND DISCUSSIONS

The study of soil agrochemical indices on plot BF 506, Beregsău area, Timiș County, led to the values presented in Table 1.

The pH values ranged from 7.66 to 8.04, the soil being characterized by a weak - moderately alkaline reaction. Mineral nitrogen (Nmin) recorded values between 9.87 mg kg^{-1} and 19.97 mg kg^{-1}, based on nitric nitrogen (NO_3^-) and ammoniacal nitrogen (NH_4^+) in soil.

Phosphorus content recorded values between 19.21 mg kg^{-1} P_2O_5 and 46.48 mg kg^{-1} P_2O_5. Potassium has recorded values in the range 325.82 and 439.13 mg kg^{-1} K_2O. Calcium content was between 7379.45-11288.23 mg kg^{-1} CaO, and magnesium had values between 1015.98-1721.35 mg kg^{-1} MgO. Sodium had values between 39.78-70.58 mg kg^{-1} Na. In the case of microelements, iron had values in the range of 12.04-25.22 mg kg^{-1} Fe, manganese oscillated between 3.53 and 6.33 mg kg^{-1} Mn, copper oscillated between 0.71 and 1.2 mg kg^{-1} Cu, zinc had values between 0.33 and 0.6 Zn, and boron had values within the range of 0.83-1.59 mg kg^{-1} B.

ANOVA test, single factor, highlighted the presence of variance in the experimental data set, in statistical safety conditions, $p << 0.001$, $F > Fcrit$, for $Alpha = 0.001$.

The correlation analysis applied to the experimental data revealed a series of positive and negative correlations, of different intensities between the agrochemical indices studied (Table 2).

Table 1. The values of soil agrochemical indices for plot BF 506

Samples	pH (H_2O)	NO_3^-	NH_4^+	Nmin	P_2O_5	K_2O	CaO	MgO	Na_2O	S	Fe	Mn	Cu	Zn	B
		(mg kg^{-1})													
BF 506-1	7.67	10.75	2.71	13.46	46.48	345.19	10689.11	1087.62	40.61	11.25	25.22	6.33	0.9	0.33	1.04
BF 506-2	7.66	9.96	1.38	11.34	25.46	357.44	9735.54	1142.32	40.76	10.36	19.86	6.16	0.8	0.38	0.83
BF 506-3	7.69	8.27	1.6	9.87	22.93	396.62	8408.73	1177.12	40.04	9.92	18.42	6.04	0.83	0.37	1.02
BF 506-4	7.97	18.34	1.63	19.97	19.21	392.33	10305.82	1090.38	44.91	11.67	16.39	3.65	0.89	0.33	1.46
BF 506-5	7.96	14.1	1.21	15.31	38.13	439.13	9293.34	1314.92	40.97	19.18	23.71	4.34	1.19	0.4	1.59
BF 506-6	7.83	10.57	1.31	11.88	38.25	418.92	7379.45	1721.35	70.58	12.31	21.68	5.3	1.2	0.59	1.47
BF 506-7	7.81	9.06	1.42	10.48	33.7	398.3	9884.45	1284.08	43.58	12.26	21.4	4.92	1.06	0.53	1.24
BF 506-8	7.85	8.77	1.44	10.21	32.96	350.51	10430.61	1109.65	41.95	13.16	16.31	4.53	0.81	0.6	1.1
BF 506-9	8.04	11.92	1.66	13.58	32.04	325.82	11288.23	1015.98	39.78	12.05	12.04	3.53	0.71	0.38	1.3

Table 2. Matrix table of correlations

	pH	NO_3^-	NH_4^+	Nmin	P_2O_5	K_2O	CaO	MgO	Na_2O	S	Fe	Mn	Cu	Zn	B
pH		0.0726	0.3551	0.1089	0.8005	0.7998	0.4462	0.8994	0.9156	0.1427	0.1526	0.0000	0.8164	0.9014	0.0126
NO_3^-	0.624		0.9749	0.0000	0.5065	0.5462	0.5634	0.7410	0.9984	0.3818	0.7531	0.0777	0.7240	0.2055	0.0637
NH_4^+	-0.350	-0.012		0.7462	0.2697	0.1512	0.2365	0.2511	0.4848	0.3246	0.5208	0.3413	0.3962	0.1883	0.3704
Nmin	0.570	0.990	0.126		0.6122	0.6825	0.4614	0.6298	0.9260	0.4681	0.8229	0.1164	0.8143	0.1425	0.0964
P_2O_5	-0.099	-0.256	0.413	-0.197		0.9529	0.9474	0.4305	0.5878	0.3145	0.0844	0.5471	0.2436	0.5159	0.7142
K_2O	0.099	0.233	-0.520	0.159	-0.023		0.0231	0.0357	0.2476	0.1479	0.1830	0.9549	0.0031	0.6157	0.0820
CaO	0.292	0.223	0.440	0.283	0.026	-0.738		0.0033	0.0346	0.9198	0.3025	0.3069	0.0688	0.3362	0.6113
MgO	-0.049	-0.129	-0.427	-0.187	0.301	0.700	-0.855		0.0012	0.5393	0.2233	0.6694	0.0052	0.0961	0.2266
Na_2O	0.042	0.001	-0.269	-0.036	0.210	0.430	-0.703	0.893		0.9642	0.6114	0.9088	0.0850	0.1152	0.2610
S	0.529	0.333	-0.372	0.278	0.379	0.524	-0.039	0.237	-0.018		0.4484	0.2504	0.0907	0.7011	0.0449
Fe	-0.519	-0.123	0.248	-0.087	0.605	0.488	-0.388	0.451	0.197	0.290		0.0801	0.0428	0.9822	0.9433
Mn	-0.973	-0.615	0.360	-0.561	0.233	-0.022	-0.385	0.166	0.045	-0.428	0.612		0.9474	0.8579	0.0410
Cu	0.091	0.138	-0.323	0.092	0.434	0.857	-0.630	0.834	0.604	0.596	0.682	0.026		0.3107	0.0590
Zn	0.048	-0.467	-0.483	-0.530	0.250	0.195	-0.364	0.588	0.562	0.149	0.009	-0.070	0.382		0.7078
B	0.783	0.639	-0.340	0.587	0.143	0.609	-0.197	0.448	0.420	0.678	0.028	-0.687	0.648	0.146	

Very high positive correlation was recorded between Nmin and NO_3^- ($r = 0.990$), and very high negative correlation was recorded between soil manganese content and pH ($r = -0.973$). High positive correlations were recorded between Cu and K ($r = 0.857$), between Cu and Mg ($r = 0.834$), respectively between Na and Mg ($r = 0.893$), and high negative correlations were recorded between Mg and Ca ($r = -0.855$).

Moderate positive correlations were recorded between B and pH ($r = 0.783$), between Mg and K ($r = 0.700$), and moderate negative correlations were recorded between Ca and K ($r = -0.738$) and between Na and Ca ($r = -0.703$). Also positive or negative correlations were recorded, lower in intensity, between other agrochemical indices studied (Table 2).

Between calcium and magnesium content of soil, respectively calcium and microelements, negative correlations were recorded, known be the antagonistic relation of calcium with the bioavailability of microelements for plants in the soils with basic reaction (Merschner, 1995).

The statistical analysis of the agrochemical indices studied showed different levels of variation, expressed by the values of coefficient of variation (CV).

The lowest variation was recorded in the case of pH (CV = 1.7802), and the largest variation was recorded in the case of nitric nitrogen NO_3^- (CV = 28.1509). In the case of ammoniacal nitrogen NH_4^+ the coefficient of variation had the value CV = 27.8444, and in the case of mineral nitrogen (Nmin), the coefficient of variation had the value CV = 24.8661. In the case of phosphorus, the value of the coefficient of variation was CV = 26.4695, and in the case of potassium CV = 9.8772. In the case of secondary macro-elements, the coefficient of variation had the values CV = 12,426 for calcium, CV = 17.4537 for Mg, CV = 21.9090 for Na and respectively CV = 21.7598 for S. In the case of microelements, the coefficient of variation recorded the values CV = 21.1678 for Fe, CV = 21.2224 for Mn, CV = 18.9461 for Cu, CV = 24.9721 for Zn and respectively CV = 20.4058 for B.

In terms of the spatial variability, compared to the analyzed surface area, based on the values of the variation coefficients, it was appreciated that the pH had the highest degree of uniformity in the characterization of the studied soil. In the case of the main macro-elements, a high degree of non-uniformity was recorded in the case of nitrogen (NO_3^-,NH_4^+, Nmin) and phosphorus, and potassium showed a higher degree of uniformity. High non-uniformity also presented the secondary macro-elements, respectively the micro-elements.

Starting from the high level of correlations, positive or negative, identified between soil agrochemical indices, the models of equations were analyzed that described the interaction of indices.

The interdependence relation between nitric nitrogen (NO_3^-) and mineral nitrogen in soil (Nmin) was most accurately described by a linear equation, relation (1), under conditions of R^2=0.981, p<<0.001, F=154.47. The graphical distribution of the Nmin values according to NO_3^-, is presented in Figure 1.

$$N\,min = 0.9983x + 1.615 \quad (1)$$

where: Nmin - mineral nitrogen in soil; x - nitric nitrogen in soil (NO_3^-).

The variation of the Mn content in the soil in relation to soil pH was described by a linear equation, relation (2), under conditions of R^2 = 0.947, p << 0.001, F = 125.52. The graphical distribution of the Mn values according to the soil pH values is presented in Figure 2.

$$Mn = -7.375x + 62.73 \quad (2)$$

where: Mn - Mn content in soil; x - soil pH

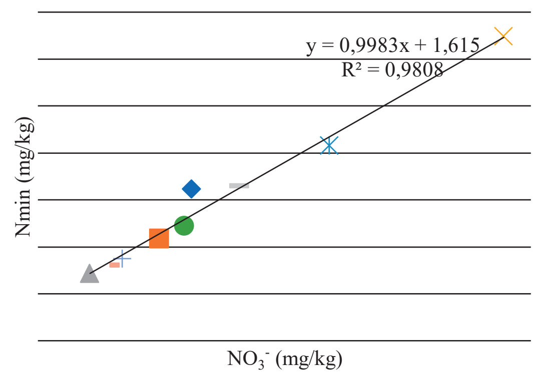

Figure 1. Graphical distribution of Nmin values in relation to NO_3^-

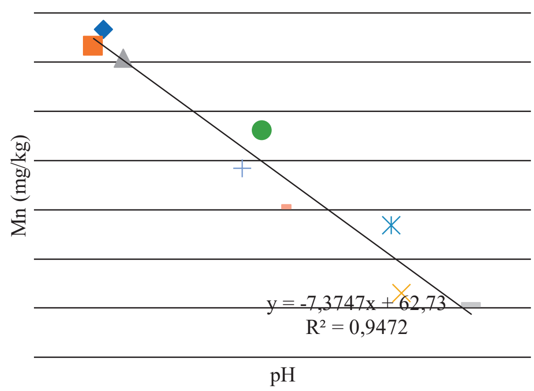

Figure 2. Graphical distribution of Mn content values according to soil pH

The variation of the Mg content in the soil with respect to the Ca content was described by a polynomial equation of degree 2, relation (3), under conditions of R^2 = 0.779, p = 0.0107, F = 10.601, and the graphical distribution is presented in the Figure 3.

$$Mg = 3.217E - 05x^2 - 0.7487x + 5412 \quad (3)$$

where: Mg - Mg content in soil; x - Ca content in soil

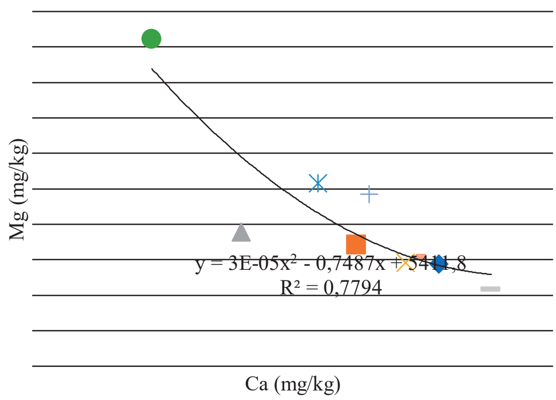

Figure 3. Graphical distribution of Mg values in relation to soil Ca content

The interrelation between Cu and K was described by a polynomial equation of degree 2, relation (4), under conditions of $R^2 = 0.768$, p = 0.012, F=9.9771. The interrelation between Cu and Mg was described by a polynomial equation of degree 2, relation (5), under conditions of $R^2 = 0.819$, p = 0.0059, F = 13.553, with graphical distribution in Figure 4.

$$Cu = 2.693E - 05x^2 - 0.01652x + 3.285 \quad (4)$$

where: Cu – Cu content in soil; x –K content in soil

$$Cu = -1.409E - 06x^2 + 0.004577x - 2.494 \quad (5)$$

where: Cu –Cu content in soil; x –Mg content in soil

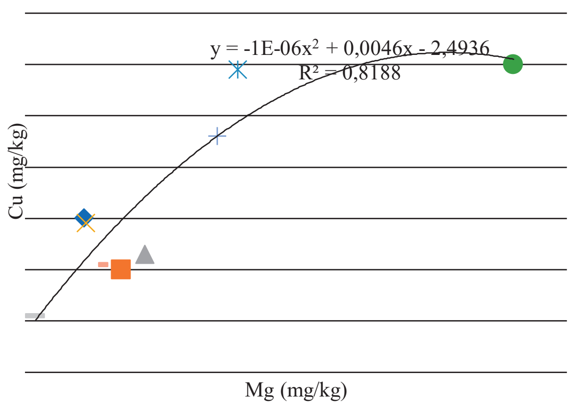

Figure 4. Graphical distribution of Cu content values according to Mg in soil

As the soil studied had a basic reaction, the interdependence relationship between Na and Ca, respectively Na and Mg, was analyzed. Were found a polynomial equation of degree 3, relation (6), which described the most faithful interdependence between Na and Ca in the soil, under conditions of $R^2 = 0.966$, p << 0.001, F = 47.957. Also was found a polynomial equation of degree 2, relation (7), that described the interdependence between Na and Mg, under conditions of $R^2 = 0.961$, p << 0.001, F = 74,151.

$$Na = -2.949E - 09x^3 + 8.648E - 05x^2 - 0.8405x + 2748 \quad (6)$$

where: Na - Na content in soil; x - Ca content in soil

$$Na = 9.034E - 05x^2 - 0.2077x + 160.1 \quad (7)$$

where: Na - Na content in soil; x -Mg content in soil

The variation of the B content according to the pH values was described by a polynomial equation of degree 2, relation (7), under conditions of $R^2 = 0.720$, p = 0.021.

$$B = -5.518x^2 + 87.9x - 348.6 \quad (8)$$

where: B - B content in soil; x - soil pH

PCA analysis explained 93.887% of variance for PC1 and 4.8938% of variance for PC2, respectively, in relation to the values of the agrochemical indices represented by the soil main macro-elements (Nmin, NO_3^-, NH_4^+, P, K). Soil samples BF 506-5, BF 506-6 and BF 506-7 were associated with P and K (P and K biplots). Soil samples BF 506-3 and BF 506-4 are more strongly associated with nitrogen (Nmin, NO_3^- and NH_4^+ biplots). The other soil samples, BF 506-1, BF 506-2, BF 506-8 and BF 506-9 showed relative independent position with respect to the soil main macro-elements (Figure 5).

In relation to the secondary macro-elements (Ca, Mg, S) and Na, PCA analysis explained 99,206% variance for PC1 and 0.79262% variance for PC2 respectively, figure 6. Soil samples BF 506-1, BF 506-7, BF 506-8 and BF 506-9 were associated with Ca (Ca biplot). Sol samples BF 506-5 and BF 506-6 were associated with Mg (Mg biplot). Soil samples BF 506-2 and BF 506-4 showed affinity for S and Na (Na and S biplots). BF 506-3 presented an independent position.

In relation to the microelements studied (Fe, Mn, Cu, Zn and B), PCA analysis explained 95,746% of variance for PC1 and 4.0768% of variance for PC2, respectively, figure 7. Soil sample BF 506-2 was strongly associated with Mn.

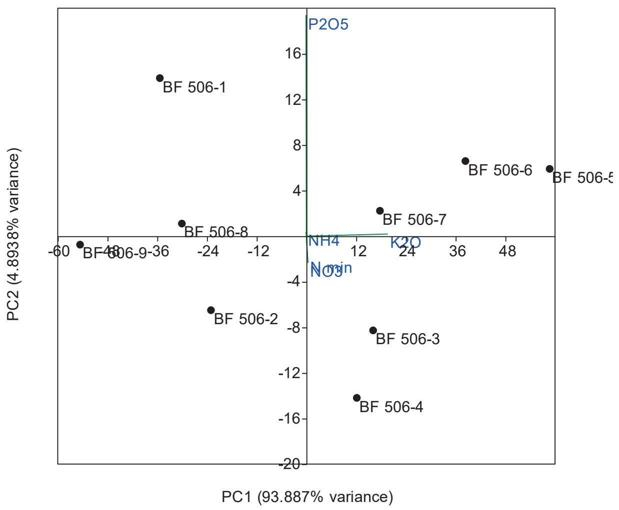

Figure 5. PCA analysis in relation to soil main macro-elements (Nmin, NO_3^-, NH_4^+, P and K)

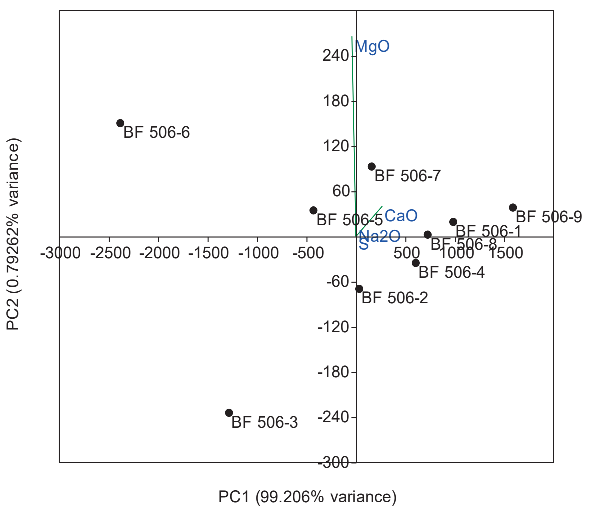

Figure 6. PCA analysis in relation to soil secondary macro-elements (Ca, Mg, S and Na)

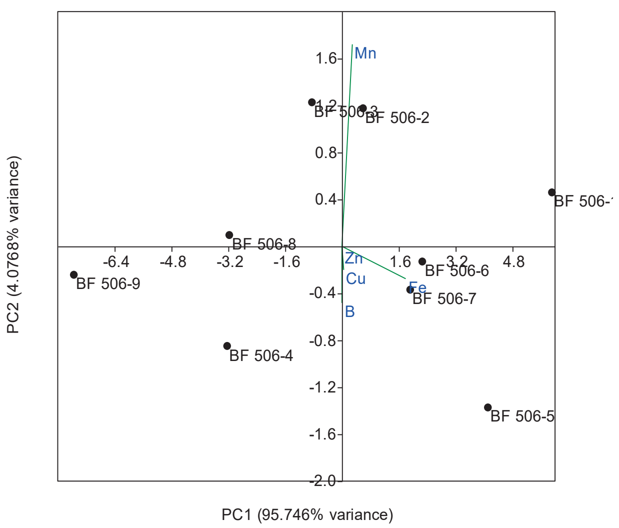

Figure 7. PCA analysis in relation to soil micro-elements (Fe, Mn, Cu, Zn and B)

Soil samples BF 506-5, BF 506-6 and BF 506-7 were strongly associated with Fe, Zn, Cu and B (Figure 7). The other soil samples, BF 506-3, BF 506-4, BF 506-8 and BF 506-9 had an independent position relative to the microelements studied (Figure 7).

Studies on soil fertility and soil-plant relationship have been conducted to establish soil health and improvement measures (Kibblewhite et al., 2007; Sigua et al., 2016; Shiau et al., 2017; Bünemann et al., 2018), for the optimization of agricultural crops fertilization (Sala and Boldea, 2011; Sala et al., 2015; 2016; Xu et al., 2015), for the improvement of agricultural technologies, the implementation and development of smart agriculture services, or for precision agriculture (Yasrebi et al., 2008; Herbei et al., 2015a; 2015b; Chen et al., 2019). The variability and high mobility of mineral nitrogen in soil has been well studied and highlighted in relation to different factors of influence, soil conditions and crop plants (Mulvaney et al., 2009; Stępień and Wojtkowiak, 2015; Nascente et al., 2017; Sharma and Bali, 2017).

Prediction models of the content or variation of mineral elements in soil have been developed for nitrogen (Bleken et al., 2009; Jégo et al., 2012; Sharifi et al., 2017; Lee et al., 2018), for phosphorus (Yang et al., 2013; Keshavarzi et al., 2015; 2016), for potassium (Phong et al., 2011; He and CHEN, 2013; Akbas et al., 2017; Laekemariam et al., 2018), for secondary macro-elements (Saggar et al., 1990; Lemos et al., 2007; Aşkın et al., 2012; Aikpokpodion et al., 2013), and for micro-elements (Grieve and Poss, 2000; Arias et al., 2005; Cheng et al., 2007; Rawashdeh and Sala, 2015a; 2015b; Huang et al., 2018).

The results obtained and communicated in the present study, are in the context of those of the specialized literature consulted, and at the same time contribute to the development of the knowledge regarding the interrelations of the nutritional elements and the agrochemical indices in the description and characterization of agricultural lands.

CONCLUSIONS

Between the agrochemical indices studied (eg soil pH, macroelements - N, P, K, secondary macroelements - S, Ca, Mg; microelements - Fe, Mn, Cu, Zn, B, Mo) interdependences were found varying levels intensity. Very high positive correlation was recorded between Nmin and NO_3^- (r = 0.990), and very high negative correlation was recorded between Mn and pH (r = -0.973).

The regression analysis facilitated the obtaining of models, in the form of linear and polynomial equations of degree 2, which described under conditions of high statistical certainty the variation Nmin in relation toNO_3^-, the variation of the content of Mn against the pH of the soil, the variation of the content of Mg with respect to Ca in soil, respectively the variation of Cu content in relation to K and Mg in soil.

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METAPOPULATION DYNAMICS AND EXTINCTION IN PRISTINE HABITATS - A DEMOGRAPHIC EXPLANATION

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Abstract

Demographic and age-structured modelling of local populations in a pool frog (Pelophylax lessonae) metapopulation reveals that the natural emigration from a population is of a magnitude which, if all emigrants are lost from the system, enhances local extinction risk in comparison to if the emigrants become immigrants in other local populations. In the latter case, annual population growth rate averaged λ = 1.2, while in the former λ averaged 0.994. Simulations indicated that single local populations in pristine habitat with emigration and no immigration have an extinction probability of 0.532 in 100 years, with extinction occurring from 11 years after isolation and mean time to extinction of 61 years. Such isolated populations were also highly vulnerable to additive mortality. In comparison, two local populations interconnected by dispersal were resilient to increased and higher local mortality provided that connectivity and the source population of immigrants were unaffected. This provides a demographic explanation of extinction patterns observed in the pool frog and other taxa that likely has general relevance for the conservation and management of subdivided populations in various landscapes.

Key words: *demographic modelling, extinction, metapopulation, Pelophylax lessonae.*

INTRODUCTION

The use of metapopulation dynamics theory (Levins, 1969; Hanski and Gilpin, 1997; Hanski and Gaggiotti, 2004) in ecology, genetics, and in biodiversity conservation and management, has increased greatly since the 1980s (e.g., Sjögren, 1988); its application to organism groups has also been discussed (e.g. Harrison, 1991; Marsh and Trenham, 2001).

In southern Sweden, the pond-dwelling pool frog (*Pelophylax lessonae*; Figure 1) occurs with a metapopulation structure along part of the Baltic Coast where local populations inhabit ponds and adjoining terrestrial habitat that together serve as “patches”. In this system, local extinction may occur deterministically due to low quality or deterioration of local patch habitat (Sjögren, 1991a; Sjögren-Gulve, 1994), but also in seemingly pristine habitats. The latter type of extinction was characterized by greater isolation from other occupied ponds, larger pond size, lower mean water temperature, closer proximity to other local extinctions, and presence of large-scale forestry between ponds; the latter also reduced (re-) colonization rates of vacant ponds (Sjögren-Gulve, 1994; Sjögren-Gulve and Ray, 1996). Sjögren-Gulve (1994) also showed that spatially correlated local extinctions were not due to a spatial correlation of habitat quality.

Figure 1. Adult female (left) and male pool frog (*Pelophylax lessonae*) from Östra Granskärsdammen, Sweden (photo: © Per Sjögren-Gulve, used by permission)

Then, what can explain this greater extinction propensity of relatively isolated local populations also in pristine habitats? And why was there still a greater extinction risk at larger pool frog ponds even though the effect of the comparably colder water temperature in larger ponds had already been taken into account in the extinction analysis (Sjögren-Gulve and Ray, 1996; Table 6.3). I set out to explore this using a combination of multiple-years field data on age-specific survival of the pool frogs, recorded dispersal between local pool frog populations (Sjögren, 1988; 1991b; Sjögren-Gulve, 1998), demographic analysis and simulation modelling.

MATERIALS AND METHODS

The population studies providing the field data and demographic data for these analyses were carried out during 1984-1992 in the landscape surrounding the village Klungsten at the northern Baltic coast of the Uppland county in southern Sweden.

Field work

The work and methods pertaining to hand-net captures, use of drift fences and individual marking of the pool frogs are described by Sjögren (1988, 1991b) and by Sjögren-Gulve (1998). Much of the results on the demography of the local population at Östra Granskärs-dammen, in focus here, are presented by Sjögren (1988; 1991b); a few have been updated due to supplementary findings and data obtained after 1988. Review and updating of results regarding dispersal rates have also been done since Sjögren-Gulve (1998).

Local samples of pool frogs (n) captured at other ponds amounted to $50 < n < 150$. In the estimation of annual age-specific (0- and 1-year olds, 2-yr-olds and adults, respectively) numbers of pool frogs that had emigrated, it was assumed that emigrants experienced the same annual mortality at the immigration locality as did non-emigrants of the same age in the source patch.

Demographic analysis

The age-specific survival and fecundity of the female pool frogs are shown in Table 1, where "-home" denotes that only individuals which returned to their natal pond the following year were included as survivors in parameter estimation. "-all" denotes that also emigrants to other ponds in the landscape were included. Sjögren (1988; 1991b) found that the average life-span did not differ significantly between males and females, but there was a tendency (p = 0.07) of higher mortality among adult females than among adult males.

Simulations and model parameterization

Simulations and their programming were carried out with the Population Viability Analysis program VORTEX ver. 10 (Lacy and Pollak, 2018) using an age-structured model. The SD of the age- and sex-specific annual survival and mortality from field data took into account observed variation during a more detailed 6-years study period, and regarding juvenile survival also considered the reproductive "failures" during cold years observed since the 1950s (Sjögren, 1991b; S. Forselius, *pers. comm.*). The SDs of the annual mortality rates used in the simulations were subtracted with the variation expected from demographic stochasticity [variance expected from a binomial distribution = $p_x(1- p_x)/(n_x-1)$] already programmed in the software, so that the entered SDs parameterizing year to year environmental variation (stochasticity) did not include demographic stochasticity too. Years with extremely cold weather caused "reproductive failure"; at worst with no pool frog larvae metamorphosing in such a year nor surviving to the next (Sjögren, 1991b). The SD of the estimated 0-yr-old mortality (p_0) in Table 1 was hence reduced so that the 99% of the variation (2.58·SD) would be within the limit of a mortality rate 1.0 at maximum. Adult pool frog survival had an opposite trend to reproductive success, probably because the frogs experience less mortality during years with cloudy and rainy weather due to less exposure to predation while basking. Thus, a negative correlation (-0.2433) between annual adult survival and reproductive success based on field data was included in all simulations.

Each simulation started with the local population(s) at stable age distribution before reproduction and no larvae nor metamorphs at start. The sequence of annual part-processes (e.g., reproduction, mortality, dispersal) in simulations was equal to the default used in the VORTEX software.

Since all emigrants found at other pool frog localities were captured closely after the annual breeding period, dispersal between the local populations in simulations was not modelled with mortality during dispersal. Females and males were assumed to be equally prone to emigrate and the slightly male-biased recap-tures of emigrants were assumed to be due mainly to their generally lower mortality than that of females of the same age (Sjögren-Gulve, 1998). Emigration and dispersal were much more frequent among juveniles and subadults than among adults (Sjögren, 1988; Sjögren-Gulve, 1998). Therefore, all scenario models with two local populations included age-differentiated dispersal among frogs up to 3 years of age; older frogs did not emigrate. These models also had an 89% correlation of the local populations' environmental variation in vital rates as quantified for correlated population fluctuations by Sjögren-Gulve (1994).

Simulations were run with a local-specific "ceiling" adjusted to consider all living frogs, also the 0-yr-old metamorphs (juveniles). This ceiling did not vary between years nor have any temporal trend. All simulation scenarios were run with 10,000 replicates each. Local extinction was defined as no frog remaining.

RESULTS AND DISCUSSIONS

With p_x-home parameter values (Table 1), which only included the survival of pool frogs that return to their natal pond the following year(s), the expected asymptotic annual growth rate (λ) of the local pool frog population became 0.994. When also including all emigrant frogs, which were found at the neighbouring pool frog locality Klubbenviken, 270 meters away sometime during the 5 years recapture study (i.e. using the p_x-all instead), the average λ became 1.20 (Table 1).

The estimated annual percentage of frogs that emigrated was 38.3% among 0-yr-olds, 5.9% among 1-yr-olds (subadults), and 1% among adults (2-yr-olds and older).

Simulations were carried out using alternative p_x values from the "home" and "all" demographic scenarios. Each alternative scenario was simulated forward for 100 years. which is often used in the general time-frame of Minimum Viable Population analyses.

Table 1. Life table for females of the local pool frog population (*Pelophylax lessonae*) at Östra Granskärsdammen

Age (x)	p_x-home	SD-home	p_x-all	SD-all	l_x-home	l_x-all	$P_{repr}(x)$	m_x	$l_x m_x$-home	$l_x m_x$-all
0	0.109	0.066	0.153	0.107	1.0	1.0	0	0	-	-
1	0.2847	0.082	0.3345	0.096	0.1090	0.1534	0	0	-	-
2	0.1968	0.042	0.2328	0.050	0.0310	0.0513	0.0283	1.46	0.0452	0.0748
3	0.2643	0,056	0.3003	0.064	0.0061	0.0119	0.6765	85.24	0.5206	1.0183
4	0.2808	0.060	0.2808	0.060	0.0016	0.0036	0.9333	157.3	0.2538	0.5642
5	0.5	0.236	0.5	0.236	0.0005	0.0010	1.0	182.5	0.0827	0.1838
6	0.5	0.236	0.5	0.236	0.0002	0.0005	1.0	206.5	0.0468	0.1040
7	0.2	-	0.2	-	0.0001	0.0003	1.0	220	0.0249	0.0554
8	0	-	0	-	0.00002	0.00005	1.0	220	0.0050	0.0111
								$R_0 = \Sigma$	0.979	2.01
								$\lambda =$	0.994	1.20

"home" denotes age-specific survival (p_x) and its standard deviation (SD) based only on the fraction of frogs which returned to their natal pond the next year. Corresponding values labelled "-all" denote estimates including both surviving emigrants and frogs that returned to their natal pond the next year. l_x is the fraction of juveniles (0-yr-olds) that survive to the age *x*. $P_{repr}(x)$ is the age-specific probability of reproduction for females, m_x is the resulting estimated average number of female offspring per female of age *x*; it was calculated from the number of eggs expected from an average-size female of age *x* multiplied by the sex ratio 0.5, $P_{repr}(x)$ (Sjögren 1991b) and estimated survival from egg to metamorph. R_0 is the net reproductive rate per generation; λ is the asymptotic population growth rate per year. Since the pool frog is a birth-pulse breeder (reproduction only c. 1 month of the entire year), *r* is not used. Female generation time (T_c) became 3.65 years

Scenarios in isolation - full emigration and no immigration

Scenarios with the age-specific survival p_x-home, m_x, emigration, no immigration, and no additional mortality, resulted in an average decline of an isolated local population and an extinction probability (P_{ext}) of 0.532 in 100 years. The variation in population trajectories is exemplified in Figure 2.

Under the premise of no added extra mortality, 47% of such local populations survived for 100 years (Figure 3).

In this scenario, the first extinctions occurred after 11 years, the mean time to extinction for the local population was 61.3 ± 21.7 years (mean ± SD) and the median 95 years, indicating that most extinctions took long time.

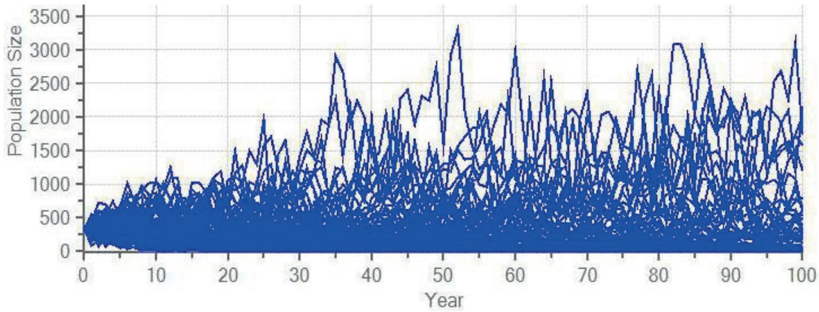

Figure 2. Population trajectories (1-yr-old and older frogs) of a subset of 100 simulation replicates of an isolated local pool frog (*Pelophylax lessonae*) population, initially with 340 frogs, when all emigrants experience mortality in the surrounding landscape ("matrix"). Asymptotic growth rate $\lambda = 0.994$ and $R_0 = 0.969$. Mean final size: 158 ± 400 (SD)

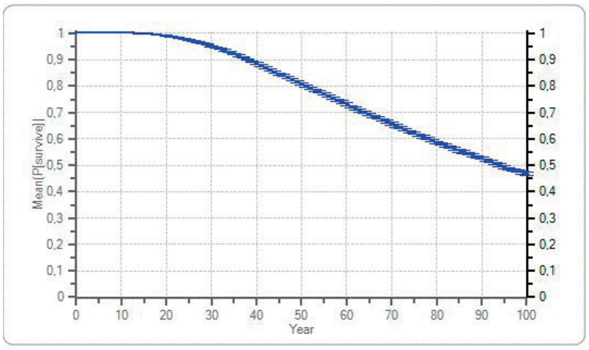

Figure 3. Yearly survival probability ± SD of an *isolated* local pool frog (*Pelophylax lessonae*) population with natural emigration and no immigration during 10,000 simulation replicates. $\lambda = 0.994$. The extinction risk in 100 years $P_{ext} = 0.532$

In this scenario, the first extinctions occurred after 11 years, the mean time to extinction for the local population was 61.3 ± 21.7 years (mean ± SD) and the median 95 years, indicating that most extinctions took long time. However, such an isolated population was also highly vulnerable to increased (additive) mortality. In a scenario with only slight additive mortality, e.g. 5% per year for all age classes, a "tipping point" appeared where the simulated local population went extinct, which began after five years, and 100% of the replicates were extinct by year 28 (Figure 4).

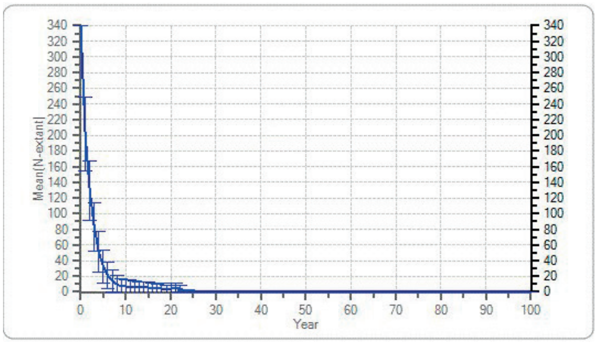

Figure 4. Mean yearly size ± SD of an isolated local pool frog (*Pelophylax lessonae*) population with natural emigration, 5% extra mortality and no immigration

This implies that anthropogenic addition of mortality to isolated local populations may have drastic negative effects on local viability and persistence, but with a time-lag.

Scenarios with two subpopulations, full emigration and connectivity

The next set of simulations examined similar scenarios as above, but for two interconnected local populations, using the p_x-all demographic data where the populations' emigration is not lost from the system.

With no added mortality, the expected mean λ of both populations was 1.2.

Even though significant and 89% correlated populations fluctuations occurred in the simulations due to spatially correlated poor reproduction during very cold years (Figure 5), none of the local populations went extinct in 100 years ($P_{ext} = 0$) provided that environmental conditions did not deviate from normal (Figure 6).

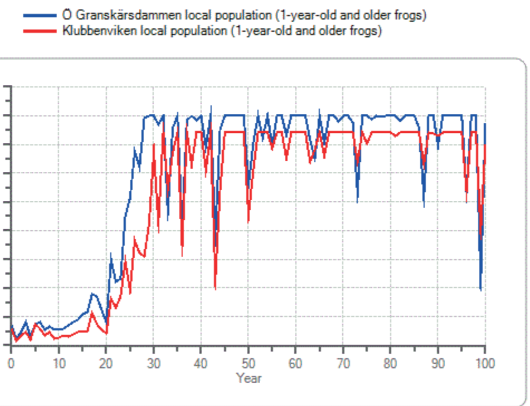

Figure 5. Population size trajectories of one simulation replicate of the two local pool frog populations Ö Granskärsdammen (blue) and Klubbenviken (red) interconnected by dispersal and with no extra mortality

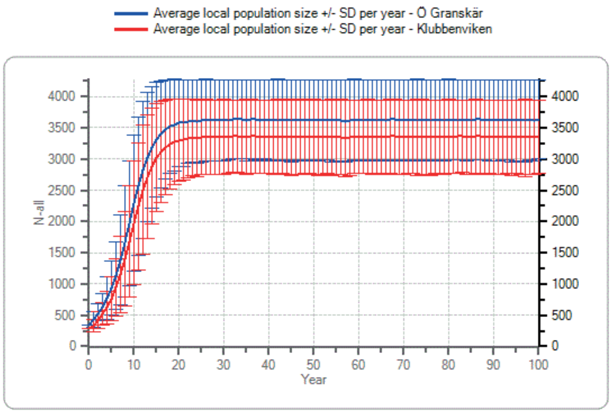

Figure 6. Yearly mean population size ± SD during 10,000 simulation replicates of the two local pool frog populations (*Pelophylax lessonae*) at Östra Granskärs-dammen and Klubbenviken, interconnected by dispersal and with no extra mortality added

Next, a number of scenarios explored the effect of various degrees of extra, additive mortality (5%, 10%, 15%, 20%, 25%, 30%) at the locality Klubbenviken while the other local population (Ö Granskärsdammen) remained unaffected. Even though the local population size at Klubbenviken became smaller in the simulations corresponding to the increased mortality, local extinction at this site did not occur at all with 5% extra yearly mortality. At 10% added mortality, local population size at Klubbenviken averaged 341 ± 1.2 (mean ± SE including 1-yr old and older frogs) during simulations and 1 local extinction occurred with recolonization from Ö Granskärsdammen the following year. With additive mortality at 15%, the Klubbenviken subpopulation averaged 222 ± 0.8 pool frogs and 4 local extinctions occurred among the 10,000 replicates with recolonization after 1 or 2 years. At 20% additive mortality, it averaged 212 ± 0.8 frogs with 5 local extinctions; at 25%, it averaged 197 ± 0.8 with 21 local extinctions. At 30% additive mortality, the subpopulation averaged 179 ± 0.8 frogs (1-yr-olds and older) and experienced 76 local extinctions among the simulation replicates (Figure 7); recolonization occurred in all cases. In this case, most if not all frogs at Klubbenviken likely are immigrants.

Summarized, these results demonstrate significantly greater resilience of demo-graphically (and genetically) interconnected sub- or local populations to withstand increased natural or anthropogenic environmental pressures in comparison to that of isolated local populations. This resilience depends on that the dispersal connectivity and the "source" population(s) are unaffected, and demonstrates "source-sink" dynamics (Pulliam 1988).

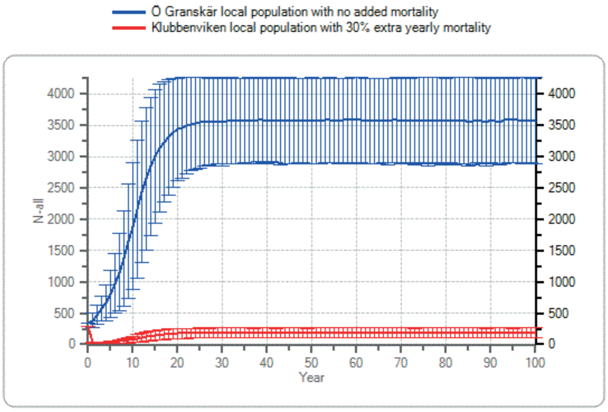

Figure 7. Yearly mean population size ± SD during 10,000 simulation replicates of the two local pool frog populations (*Pelophylax lessonae*) at Östra Granskärs-dammen and Klubbenviken, interconnected by dispersal and with 30% extra mortality at Klubbenviken

Already more than 30 years ago, scientists pointed to the need to distinguish between "source" and "sink" habitats in field studies of populations. This is important in both management and conservation. In his seminal paper, Pulliam's (1988) argued and showed that active dispersal from source habitats can maintain even large "sink populations" and, furthermore, that such dispersal may be evolutionarily stable in the long term.

The second lesson (Lesson 2) from this case is that if emigration continues as normal, a local population in a pristine pond or patch that becomes isolated due to local extinction of neighbouring populations may still persist for quite a long time but faces a much higher extinction risk, similar to an "extinction debt" (Kuussaari et al. 2009). Since habitat quality *per se* was not spatially correlated in the Swedish metapopulation system, this increased extinction risk may also explain the spatially correlated extinction pattern in the pool frog metapopulation (Sjögren-Gulve, 1994). The extinction of a neighbouring "source" population may unfold a much higher local vulnerability of remaining populations.

Lesson 3 is that the results imply a risk of a "tipping point" phenomenon, in which isolated local populations may go extinct quite rapidly

since isolation commenced, due to only modestly increased or added mortality (Figure 4). Thus, introduction of predatory fish such as pike (*Esox lucius*) is detrimental (Sjögren-Gulve, 1994; Sjögren-Gulve and Ray, 1996), especially into ponds with source populations.
Altogether, the results provide a demographic explanation of the isolation-associated extinction pattern observed in the Swedish pool frog metapopulation and also in other taxa.

Other amphibians
Regarding other European amphibians, Laan and Verboom (1990) examined the number of species and their presence/absence patterns in old vs. recently constructed pools in the Netherlands in relation to characteristics of the pools and the surrounding landscape. In both old and new pools, species number was positively correlated with vicinity of a wood, which was interpreted as a connectivity element in the landscape. Pool age was the prime predictor of species number in the new pools, and differences in pond colonization rate were related to the species' abundance, suggesting that dispersal was important in compensating for local extinctions in these local frog communities.
The above interpretation was corroborated by a field experiment by Mazerolle and Vos (2006) who presented edible frogs (*Pelophylax* klepton *esculenta*) with a choice between a short route through hostile environment and a longer but safer route to reach their focal pond. The frogs' ability to orient toward the pond decreased with pond distance and increased with distance to a hedgerow. Mazerolle and Vos concluded that both landscape and weather variables influence the movements of amphibians in agricultural settings, and that landscape quality thus can be important for population persistence.
In many European countries, the tree frog *Hyla arborea* has declined due to habitat fragmentation and loss of suitable breeding sites. Auffarth et al. (2017) predicted the viability of a *Hyla arborea* population of c. 70 adults inhabiting an isolated pond in the region of Hannover (Germany), by combining life history data with genotypic information from microsatellite markers. Their findings are in agreement with results in this pool frog analysis. Auffarth et al. found a high probability of extinction within the next 50 years, with juvenile survival being a crucial demographic parameter for population persistence. Simulated natural immigration, or population supplementation, prevented genetic erosion and markedly increased the probability of population survival. They concluded that future management interventions should improve the pond habitat to enhance survival at young life stages, and create migration corridors to facilitate connectivity with adjacent local populations and/or consider translocation of individuals.
Similar conclusions were made for *Hyla arborea* populations in Switzerland by Angelone et al. (2011). They analysed how functional connectivity (frog movement and gene flow) among local populations depends on the landscape matrix between occupied habitat patches. They examined the effects of landscape elements and geographic distance on genetic differentiation among the local populations. Hedgerows and various structure-rich landscape elements affected gene flow positively. At distances < 2 km, only a larger river acted as a barrier to gene flow. At distances > 2 km, geographic distance had a negative effect on gene flow as had landscape elements such as forests and roads.
Findings by Ficetola and Bernardi (2004) are also in agreement with results presented here. They analysed patterns in amphibian presence/absence during the breeding season in 84 wetlands in Northern Italy in relation to their features and isolation. They found that amphibian presence depended strongly on habitat quality and isolation: the richest communities were found in fish-free, sunny wetlands near other occupied wetlands (cf. Lesson 1); the negative effects of isolation were not biased by spatial autocorrelation of habitat features. The system showed strong species nestedness, with species persistence dependent on the contemporary effects of species adaptability and mobility. The commonest species, the edible frog (*Pelophylax* klepton *esculenta*) and the Italian tree frog (*Hyla intermedia*) readily move through the landscape matrix using canals and hedgerows, and maintain metapopulations across the landscape, while the rarest species (newts and toads) are more sensitive to habitat change and

exhibited strong isolation effects (cf. Lessons 1-3). Ficetola and Bernardi concluded that if human exploitation of the studied landscape continues without consideration of such species groups, only the mobile and opportunistic few species will persist.

In population simulations, Griffiths (2004) found that local populations of the great crested newt (*Triturus cristatus*) - also a species protected by the EU Habitats Directive (Council Directive 92/43/EEC) - had an annual λ of 1.12. The extinction risk declined with an increasing number of local populations in the metapopulation. In scenarios with no dispersal, at least 4 local populations with 200 newts each were required for system persistence times > 100 years. With interpopulation dispersal, similar persistence times could be achieved with a similar number of local populations, but then even if they were half as big (cf. Lesson 1).

Roads are one anthropogenic factor in the landscape that affects amphibians and other taxa (Elzanowski et al., 2009; Selva et al., 2011).

That emigrant survival is important, indirectly sheds light on results from a study by Lesbarrères et al. (2006) of the agile frog (*Rana dalmatina*) in France. They analysed the degree of genetic diversity and differentiation within and among seven local populations far from trafficked roads ("non-fragmented") in France compared to that of four local populations situated pairwise on both sides of a major highway ("fragmented"). The landscapes surrounding the agile frog ponds were otherwise similar and no ecological pond habitat variables (e.g. pond size) differed significantly between the groups. The four fragmented local populations had much lower genetic variation and greater differentiation than those of the non-isolated populations, and their local population size was less than 1/3 of the average of the non-isolated populations. Viewed in light of the conclusions about significant mortality in amphibians caused by road traffic, for example by Elzanowski et al. (2009), Lesbarrères et al.'s (2006) results indicate that the fragmented agile frog populations likely have become much smaller and more depauperate in genetic variation because of high mortality among their emigrants due to the trafficked road (cf. Figure 7 and Lessons 1-2).

A conservation example with amphibians in North America where this study's Lessons 1 and 3 may apply is provided by Fellers and Drost (1993). They found that healthy, seemingly well-protected populations of the Cascades frog (*Rana cascadae*) - which from historic accounts and museum records was once abundant close to the Lassen Volcanic National Park in California (USA) - had disappeared for no obvious reason. Where biologists less than 15 years earlier found 40 or more frogs at some of the examined sites, Fellers and Drost located two frogs at a single locality. This decline seemed caused by a combination of the presence of non-native predatory fish (i.e. extra mortality), which restricted habitat and limited dispersal of the frogs, loss of breeding habitat due to a five-year drought, and a gradual loss of open meadows and associated aquatic habitats.

In another example, Harper et al. (2008) used model projections of wood frog (*Rana sylvatica*) and spotted salamander (*Ambystoma maculatum*) populations and related the amount of high-quality terrestrial habitat surrounding isolated wetlands to the decline and risk of extinction of the local amphibian populations. The simulation results indicated that current U.S. state-level wetland regulations were inadequate to support viable populations of the pool-breeding amphibians. They found that species with different life-history strategies responded differently to the loss and degradation of terrestrial habitat. The wood frog, with a short life span and high fecundity, was most sensitive to habitat loss and isolation, whereas the longer-lived spotted salamander with lower fecundity was most sensitive to habitat degradation which reduced adult survival rates. Their results demonstrated that high local population persistence requires sufficient terrestrial habitat, maintenance of habitat quality, and connectivity among the local populations. They thus stressed the essential roles of adequate terrestrial habitat and connectivity for maintenance of wetland biodiversity and ecosystem function.

Other taxonomic groups

To what extent may findings from studies on amphibians apply also to other taxonomic groups? Mazerolle and Villard (1999) reviewed 61 studies which simultaneously examined the effects of landscape variables and patch variables on the presence/absence and abundance of some invertebrate taxa, amphibians, reptiles, birds and mammals in various landscapes. They found that patch habitat variables had significant effects on all examined groups in all landscape types. Landscape variables, such as area of suitable habitat within a certain distance, were significant predictors of presence/absence for the vertebrate species but not for the majority of the invertebrates. Mazerolle and Villard (1999) concluded that more consideration of landscape characteristics generally is likely to enhance strategies for species conservation in landscapes.

As likely were the case for the Klubbenviken pool frog population under higher additive mortality, the review by Millon et al. (2019) found that the number of immigrants exceeded locally born individuals in recruitment for most avian populations (median = 0.57 based on 37 studies); this was twice that estimated for mammalian populations (median = 0.26; 11 studies). They argued that overall, it is likely that most populations benefit from immigrants (cf. Lesson 1) without necessarily being "sink" populations and that quantitative estimates of immigration should be core to future demographic studies in various landscapes.

In a review of 432 papers - most of them focusing on birds, mammals, and forested systems - Heinrichs et al. (2019) found that source-sink theory has become increasingly relevant for species conservation and management. While 79% of the reviewed papers claimed to identify source-sink dynamics in these taxa - indicating potential relevance of the demographic explanation here - nearly 23% of the 432 studies used neither demographic nor movement metrics to make their conclusions. Heinrichs et al. stressed that future studies need to take a more rigorous approach to defining sources and sinks to better assess the prevalence of source-sink dynamics (cf. Lessons 1-3) in systems subject to management and/or conservation.

Bommarco et al. (2014) examined species richness of habitat specialist and generalist butterflies, bees, hoverflies, and vascular plants in 45 seminatural grassland fragments of various size and degree of connectivity; those were situated in landscapes with contrasting land use conversion history. Habitat loss was estimated by comparing modern maps to ~45 years old aerial photographs. They found that bees responded rapidly to habitat loss, possibly because their primary nesting resource was destroyed. Species richness of specialist plants was best explained by historical habitat connectivity, richness of hoverflies by historical habitat area, and richness of butterflies by both historical habitat area and connectivity, indicating extinction debt for all these taxa (cf. Lessons 1-2). Habitat generalist butterflies and hoverflies, but not plants and bees, exhibited extinction debt mainly in relation to habitat area. No effect of landscape type *per se* was found.

They hypothesized that the slow extinctions (cf. Lesson 2) of persistent and long-lived plants might explain extinction debt for both plants and the herbivorous insects linked to these plants.

CONCLUSIONS

Amphibians are an important part of ecosystems and food webs (e.g. Gibbons et al., 2006).

This paper provides a demographic explanation of the isolation-associated extinction pattern observed in the Swedish pool frog metapopulation. Much indicates that the explanation has general relevance for the viability, conservation and management of subdivided populations of multiple organism groups - in forest, agricultural and mixed landscapes.

For several species of animals and plants, including amphibians, Bell et al. (2019) found that gene flow through immigration has helped prevent population extinction. Yet, they found that augmented gene flow is rarely used as a conservation strategy. In conservation actions for small isolated populations, they thus advocated that actions' focus should shift away from managing populations in isolation, and toward widespread restoration of gene flow.

In their recent review, Millon et al. (2019) found that a clear picture is still missing of how widely the immigration rate varies both among- and within-populations, in relation to extrinsic and intrinsic ecological conditions, even for the best studied avian and mammalian populations. They found that this empirical knowledge gap precludes the emergence of a sound conceptual framework that is important for conservation and population ecology.

In conclusion, my results and reviewed material confirm the significance of population connectivity, dispersal and gene flow in species conservation and in the landscape management to "protect, conserve and enhance the Union's natural capital" (e.g. European Commission 2013). From an overarching "biodiversity, ecosystems, ecosystem services and nature-based solutions" standpoint, it is of key importance to identify and conserve source populations, and to restore "sink" populations and/or connectivity wherever it is relevant.

Most landscapes are under management in Europe, and management is under responsibility. Article 10 of the EU Habitats Directive (Council Directive 92/43/EEC) states that "*Member States shall endeavour, where they consider it necessary, in their land-use planning and development policies and, in particular, with a view to improving the ecological coherence of the Natura 2000 network, to encourage the management of features of the landscape which are of major importance for wild fauna and flora. Such features are those which, by virtue of their linear and continuous structure (such as rivers with their banks or the traditional systems for marking field boundaries) or their function as stepping stones (such as ponds or small woods), are essential for the migration, dispersal and genetic exchange of wild species.*"

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GROWTH SIGNS OF *Nymphaea candida* IN VARIOUS ECOLOGICAL AND CENOTIC CONDITIONS OF DESNA BASIN (UKRAINE)

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Abstract

Nymphaea candida J. et C. Presl. is a relict species which is a typical representative of an ecological group of attached aquatic higher plants with floating leaves. The conducted research was meant to evaluate the indicators that characterize the Nymphaea candida growth in different conditions of the Desna River basin and to find out the influence of the leading ecological-cenotic factors on them. Based on the analysis of 12 dynamic metric morphological parameters values and five dynamic allometric values, the information on Nymphaea candida plant growth rate in six communities is provided. The influence on the growth of such environmental factors such as the phytocoenoses Total plant cover, water column, its transparency andnature of bottom sediments was evaluated. The water colum and phytocoenoses overall projective coverage showed statistically significant effects, with a force of 54.4-99.0% and 23.6-90.0%, respectively, on all dynamic morphoparameters of Nymphaea candida plants. The bottom sediments did not show a statistically significant effect on only one morphoparameter, and the factor influence force on the dimensional features of Nymphaea candida plants mainly varies within 8.7-89.5%. Water transparency did not show a statistically significant effect on the three morphological parameters, given the influence of this factor on all other features at the level of 13.2-19.4%. In Nymphaea candida plants, the largest majestic significant proportion (58.8%) of the dynamic morphoparameters were in the population of Nymphaea candida - Potamogeton lucens community, the smallest (in 64.7%) were in Numphoides peltata - Ceratophyllum demersum. The fastest growth of Nymphaea candida plants is in areas where there is no flow, the water columnvaries within 50-100 cm, the water transparency reaches the bottom, the silt bottom sediments are represented, and the phytocoenoses total projective coverage is 60-85%. Nevertheless, the total plant cover of Nymphaea candida can be from 10% to 40%. Within the study area, such habitat conditions are optimal to ensure the continued existence of populations of this species as a whole.

***Key words**: population, growth, dynamic morfoparametry, environmental coenotic factors, Nymphaea candida.*

INTRODUCTION

Nymphaea candida J. et C. Presl. is a typical representative of an ecological group of attached aquatic higher plants with floating leaves (Heidan, 1965; Dubyna, 1975; 2006; Dubyna et al., 1993). In the study region, this species grows in river beds, bays, floodplains, reservoirs. In the composition of aquatic phytocenoses, it is quite often the dominant or co-dominant (Dubyna, 1982). However, due to the steadily increasing negative anthropogenic impact on aquatic ecosystems, *Nymphaea candida* is becoming less common. In some regions of Ukraine, in particular in the Sumy region, it is already included in the list of species in need of special protection (http://knt.sm.gov.ua/).

Considering that *Nymphaea candida* is a relict, and phytocoenoses with the participation of this species successfully perform ecologically-stabilizing functions (water treatment, energy storage, conservation and biogeochemical), the topical issue is its conservation at the population level (Dubyna, 1982; Zlobin, 1992). However, at present, the population level of *Nymphaea candida* has not been sufficiently studied. An important component of the development of a set of approaches based on science concerning the organization of *Nymphaea candida* populations' active protection is the study of features and patterns of growth of individuals in different ecological-cenotic conditions.

In general, growth is an integral phenomenon that reflects the level and ratio of all physiological and biochemical processes that occur in plants. Besides, growth is the best indicator of the individuals' vitality level (Zlobin et al., 2009). It is not by chance that scientists pay considerable attention to the estimation of growth processes in plants. However, meantime, such studies have largely covered terrestrial plants (Morozova, 2009; Scherbakova & Novosad, 2013; Skliar & Zlobin, 2013; Klymenko & Skliar, 2015) and aquatic plants have been covered very little. There are some papers dedicated to the estimation of growth parameters in *Trapa natans* L. sl (Skliar & Skliar, 2017) and *Potamogeton natans* L. (Skliar, 2017).

The research was aimed to evaluate the indicators characterizing the growth of *Nymphaea candida* in different conditions of Desna basin and to find out the influence of the leading ecological-cenotic factors on them.

MATERIALS AND METHODS

The basis of the presented publication is grounded on the results of researches carried out in the reservoirs of Desna basin in six communities of higher aquatic plants, which differ significantly from each other in the complex of ecological-cenotic features (Table 1). There were studied as typical (No. 3, 5, 6) so as less common communities in the region (No. 1, 2, 4). The set of selected sites forms a complex ecological-cenotic gradient where the water column, its transparency, the bottom sediments, the total plant cover of the community, etc. are changing.

Table 1. Ecological-coenotic characteristics of habitats of *Nymphaea candida* coenopopulations

№ Of the water reservoir (habitat)	Community	Average water column, cm	Flow	Water transparency, cm	Bottom sediments	Total plant cover, %
1	*Nymphoides peltata - Ceratophyllum demersum*	40	absent	To the bottom	sandy silt	100
2	*Nymphaea candida purum*	60	absent	To the bottom	silty	60
3	*Nuphar lutea - Ceratophyllum demersum*	90	absent	To the bottom	silty	85
4	*Nymphaea candida - Potamogeton lucens*	100	absent	To the bottom	silty	85
5	*Nuphar lutea purum*	120	absent	90	silty	80
6	*Nuphar lutea + Potamogeton natans*	140	absent	75	silty	70

According to conventional approaches (Hejny, 1960; Belavskaya, 1982; Dubyna, 1982; Skiar, 2006), in each community, there were made geobotanical descriptions, which were accompanied by an assessment of the species composition of the phytocenosis, their abundance and projective coverage, as well as water column, water flow, water transparency and nature of bottom sediments.

To estimate the growth rates of *Nymphaea candida*, 20-25 plants of this species in each population were studied, in which the complex of size indicators such as total phytomass (W), leaf phytomass (WL), leaf number (NL), leaf area (A), length of the petiole (H), mass (Wg) and number (Ng) of generative organs were evaluated. As predicted by the method of estimation of growth indicators, the account of these characteristics was performed twice during the period of intensive plant growth with an interval between measurements of 10-14 days.

Based on these calculations, we determined the values of dynamic morphological parameters, which, in turn, according to conventional approaches (Hunt, 1978; Zlobin, 1989; Zlobin et al., 2009), were divided into two groups:

a) metric group which provides information on the change rate of the size of a single metric indicator in ontogeny (Table 2);

b) allometric group which reflects the change rate of allometric ratios in ontogeny (Table 3).

Table 2. List of dynamic metric morphological parameters that were used to estimate *Nymphaea candida* plant growth

Morphoparameters	Symbols and formulas of morphoparameters[1]	Units of measurement
Absolute growth rate indicators		
Absolute growth rate of total phytomass	$AGR = (W_2 – W_1) / \Delta T$	g/day
Absolute growth rate of the total mass of leaves	$AGRWL = (WL_2 – WL_1) / \Delta T$	g/day
Absolute speed of leaf area formation	$AGRA = (A_2 – A_1) / \Delta T$	cm^2/day
Absolute growth rate of length	$AGRH = (H_2 – H_1) / \Delta T$	cm/day
Absolute speed of leaf formation	$AGRNL = (NL_2 – NL_1) / \Delta T$	pcs./day
Absolute growth rate of generative organs mass	$AGRWg = (Wg_2 – Wg_1) / \Delta T$	g/day
Absolute speed of generative organs formation	$AGRNg = (Ng_2 – Ng_1) / \Delta T$	pcs./day
Relative growth rate indicators		
Relative growth rate of total phytomass	$RGR = (\ln W_2 – \ln W_1) / \Delta T$	g/day
Relative growth rate of length	$RGRh = (\ln H_2 – \ln H_1) / \Delta T$	cm/day
Relative growth rate of the total mass of leaves	$RGRWL = (\ln WL_2 – \ln WL_1) / \Delta T$	g/day
Relative speed of leaf area formation	$RGRA = (\ln A_2 – \ln A_1) / \Delta T$	cm^2/day
Relative speed of leaf formation	$RGRNL = (\ln NL_2 – \ln NL_1) / \Delta T$	pcs./day

[1]Here and in Table 3, the lower index "1" indicates the results of the first dimension measurement; the lower index "2" indicates the results of the second dimension measurement; ΔT is the time between the first and second measurements.

Table 3. List of dynamic allometric morphoparameters that were used to estimate *Nymphaea candida* plant growth

Morphoparameters	Symbols and formulas of morphoparameters[1]	Units of measurement
Net-assimilation	$NAR1 = \frac{W_2 – W_1}{\Delta T} \times \frac{\ln A_2 – \ln A_1}{A_2 – A_1}$	$g/cm^2/day$
	$NAR2 = \frac{2 (W_2 – W_1)}{(A_2 + A_1) \Delta T}$	$g/cm^2/day$
Productivity of a leaf area forming	$LAR1 = \frac{A_2 – A_1}{\Delta T} \times \frac{\ln W_2 – \ln W_1}{\ln A_2 - \ln A_1}$	$cm^2/g/day$
	$LAR2 = \frac{A_2 – A_1}{\Delta T} \times \frac{\ln W_2 – \ln W_1}{W_2 – W_1}$	$cm^2/g/day$
	$LAR3 = \frac{A_2 – A_1}{W_2 - W_1} \times \frac{\ln W_2 – \ln W_1}{\ln A_2 - \ln A_1}$	$cm^2/g/day$

To evaluate the influence of the leading ecological-cenotic factors (water column, total plant cover, water transparency) on the values of the dynamic morphoparameters of *Nymphaea candida*, the dispersive analysis there was computed, accompanied by calculations of power of influence (Tsarenko et al., 2000).

RESULTS AND DISCUSSIONS

The values of the dynamic morphoparameters characterizing the growth rate processes in *Nymphaea candida* in the studied cenopopulations are shown in Table 4.

Dynamic metric morphoparameters. The highest absolute growth of phytomass (AGR) is observed in *Nymphaea candida* individuals in *Nymphaea candida - Potamogeton lucens* (32.7±3.99 g/day) and *Nuphar lutea - Ceratophyllum demersum* (25.4±3.75 g/day) communities. The lowest AGR value is observed in the cenopopulation of *Nymphoides peltata - Ceratophyllum demersum* community (1.9±0.47 g/day). In other cenopopulations, the growth of phytomass varies from 5.3±1.22 g/day to 13.3±3.73 g/day (Figure 1).

The values of absolute speed of leaf formation (AGRNL), absolute growth rate of its total mass (AGRWL), absolute growth rate of generative organs mass and number of generative organs (AGRWg and AGRNg, respectively), are the largest in *Nymphaea candida - Potamogeton lucens* individuals (0.43±0.045 pcs./day, 8.7±0.99 g/day, 3.4±0.55 g/day, 0.22±0.016 pcs./day, respectively), the smallest in *Numphoides peltata - Ceratophyllum demersum* individuals (0,10±0.012 pcs./day, 0.6±0.14 g/day, 0.3±0.08 g/day, 0.03±0.006 pieces/day, respectively). In other populations, the magnitudes of AGRNL parameters range from 0.13±0.008 pcs./day to

0.31±0.037 pcs./day, AGRWL - from 1.5±0.17 g/day to 8.3±1.26 g/day, AGRWg - from 0.4±0.06 g/day to 1.6±0.51 g/day, AGRNg - from 0.06±0.006 pcs./day to 0.13±0.025 pcs./day.

The highest absolute growth rate of leaf area (AGRA) is in individuals of *Nuphar lutea - Ceratophyllum demersum* community (190.8±28.67 cm²/day), the smallest in *Numphoides peltata - Ceratophyllum demersum* community (12.8±2.77 cm²/day). In other populations, the AGRA parameter ranges from 24.3±2.86 cm²/day to 142.3±16.23 cm²/day.

Table 4. Dynamic morphoparameters of *Nymphaea candida* in different ecological-coenotic conditions of the Desna basin reservoirs

Morphoparameters	Communities					
	Nuphar lutea + Potamogeton natans	*Nuphar lutea - Ceratophyllum demersum*	*Nymphaea candida subpurum*	*Nymphaea candida - Potamogeton lucens*	*Nuphar lutea subpurum*	*Nymphoides peltata - Ceratophyllum demersum*
	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$
Dynamic metric morphoparameters						
AGR	5.8 ± 0.59	25.4 ± 3.75	13.3 ± 3.73	32.7 ± 3.99	5.3 ± 1.22	1.9 ± 0.47
AGRWL	1.5 ± 0.17	8.3 ± 1.26	4.7 ± 1.33	8.7 ± 0.99	1.6 ± 0.33	0.6 ± 0.14
AGRNL	0.13 ± 0.008	0.30 ± 0.028	0.31 ± 0.037	0.43 ± 0.045	0.14 ± 0.018	0.10 ± 0.012
AGRWg	0.4 ± 0.06	1.4 ± 0.31	1.6 ± 0.51	3.4 ± 0.55	0.6 ± 0.15	0.3 ± 0.08
AGRNg	0.06 ± 0.006	0.12 ± 0.027	0.13 ± 0.025	0.22 ± 0.016	0.07 ± 0.011	0.03 ± 0.006
AGRH	1.4 ± 0.05	2.2 ± 0.08	1.7 ± 0.06	2.4 ± 0.04	1.2 ± 0.05	0.7 ± 0.04
AGRA	24.3 ± 2.86	190.8 ± 28.67	99.2 ± 27.90	142.3 ± 16.23	29.8 ± 6.15	12.8 ± 2.77
RGR	0.06 ± 0.001	0.09 ± 0.002	0.10 ± 0.004	0.11 ± 0.002	0.06 ± 0.003	5.20 ± 0.210
RGRA	0.07 ± 0.001	0.12 ± 0.002	0.13 ± 0.004	0.14 ± 0.002	0.07 ± 0.002	7.10 ± 0.190
RGRWL	0.04 ± 0.001	0.08 ± 0.002	0.08 ± 0.004	0.09 ± 0.001	0.05 ± 0.002	0.04 ± 0.001
RGRNL	0.02 ± 0.001	0.04 ± 0.001	0.04 ± 0.001	0.05 ± 0.001	0.03 ± 0.001	2.40 ± 0.111
RGRh	0.05 ± 0.001	0.07 ± 0.001	0.07 ± 0.001	0.08 ± 0.001	0.05 ± 0.001	0.74 ± 0.040
Dynamic allometric morphoparameters						
NAR1	0.02 ± 0.001	0.04 ± 0.001	0.01 ± 0.001	0.04 ± 0.007	0.01 ± 0.001	0.01 ± 0.001
NAR2	0.006 ± 0.0001	0.009 ± 0.0001	0.004 ± 0.0003	0.009 ± 0.0001	0.004 ± 0.0001	0.002 ± 0.0001
LAR1	20.6 ± 2.49	166.3 ± 23.28	77.9 ± 22.58	122.6 ± 14.23	23.9 ± 5.15	9.8 ± 2.26
LAR2	0.21 ± 0.006	0.35 ± 0.050	0.70 ± 0.042	0.45 ± 0.099	0.31 ± 0.010	0.27 ± 0.012
LAR3	2.8 ± 0.07	2.9 ± 0.37	5.4 ± 0.29	3.3 ± 0.71	4.2 ± 0.14	4.4 ± 0.16

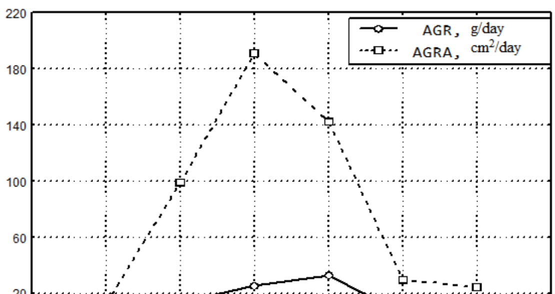

Figure 1. Dynamics of absolute growth rate of phytomass (AGR - g/day) and absolute growth rate of leaf area (AGRA - cm²/day) in populations of *Nymphaea candida* on a water column gradient (the numbering of the habitats corresponds to the Table 1)

In *Numphoides peltata - Ceratophyllum demersum* community, compared to *Nymphaea candida - Potamogeton lucens* community, the value of the AGR morphoparameter was decreased 17.2 times, AGRWL - 14.5 times, AGRNL - 4.3 times, AGRWg - 11.3 times, AGRNg - 7.3 times, and the magnitude of AGRA, compared to its largest value in the population community of *Nuphar lutea–Ceratophyllum demersum* - 14.9 times.

Factors of water column (depth), total plant cover, and bottom sediments showed statistically significant influence on all morphoparameters that characterize the absolute growth rate, influencing the levels of 63.4-94.6%, 23.6-53.4% and 12.6-46.9% (Table 5). The water transparency factor did not show a statistically significant influence on one indicator of absolute growth rate; for all others its floating power is 13.2-18.9%. The leading factor in determining the patterns of variation in absolute growth rate in *Nymphaea candida* is a water column.

The magnitudes of the morphoparameters of the relative growth rate of total phytomass (RGR) and leaf area (RGRA), respectively, range from 0.06±0.003 g/g/day to 5.20±0.210 g/g/day and from 0.07±0.002 cm²/cm²/day to

7.10±0.190 cm²/cm²/day (Figure 2). They are the highest in the population community of *Numphoides peltata - Ceratophyllum demersum*, while the smallest are in *Nuphar lutea subpurum* and *Nuphar lutea + Potamogeton natans* individuals.

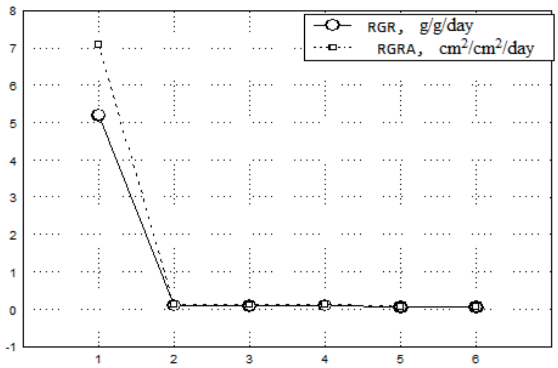

Figure 2. Dynamics of relative growth rates of phytomass (RGR - g/g/day) and relative growth rate of leaf area (RGRA - cm^2/cm^2/day) in *Nymphaea candida* cenopopulations on a water column gradient (the numbering of the habitats corresponds to the Table 1)

According to the values of relative growth rate of leaf phytomass (RGRWL), populations are divided into two groups. One includes coenopopulations of *Nymphaea candida - Potamogeton lucens*, *Nymphaea candida subpurum* and *Nuphar lutea - Ceratophyllum demersum* communities. They are characterized by relatively high RGRWL values (0.08±0.004 - 0.09±0.001 g/g/day). The second group includes coenopopulations of *Nuphar lutea subpurum*, *Nuphar lutea + Potamogeton natans* and *Numphoides peltata - Ceratophyllum demersum*. The values of RGRWL are smaller in 1.6-2.3 times and there are 0.04±0.001-0.05±0.002 g/g/day. The highest value of this indicator is in the individuals of the coenopopulations of the *Nymphaea candida - Potamogeton lucens* community, and the lowest is in the *Nuphar lutea + Potamogeton natans* and *Numphoides peltata - Ceratophyllum demersum* individuals. The highest values of the relative leaf formation rate (RGRNL) are found in the individuals of the *Numphoides peltata - Ceratophyllum demersum* community (2.40±0.111 pcs./pcs./day). The value of RGRNL in other four populations (in communities *Nuphar lutea subpurum, Nuphar lutea - Ceratophyllum demersum, Nymphaea candida subpurum, Nymphaea candida - Potamogeton lucens*) ranges from 0.03±0.001 pcs./day to 0.05±0.001 pcs./day. The lowest value of this morphoparameter (0.02±0.001 pcs./day) was detected in the community *Nuphar lutea + Potamogeton natans*.

Table 5. Influence of ecological-coenotic factors on the magnitude of morphoparameters of *Nymphaea candida*[1]

Morphparameter	Water column			Total plant cover			Bottom sediments			Water transparency		
	F-test	Confidence level	Factor influence force,%	F-test	Confidence level	Factor influence force,%	F-test	Confidence level	Factor influence force,%	F-test	Confidence level	Factor influence force,%
Dynamic metric morphoparameters												
AGR	26.0	0.0000*	75.2	6.8	0.0007*	31.1	10.6	0.0021*	18.4	4.1	0.0237*	15.0
AGRWL	21.9	0.0000*	71.8	7.1	0.0005*	32.2	10.7	0.0020*	18.5	5.0	0.0113*	17.7
AGRNL	26.0	0.0000*	75.1	9.5	0.0005*	38.8	12.5	0.0009*	21.0	5.3	0.0085*	18.7
AGRWg	14.9	0.0000*	63.4	4.6	0.0066*	23.6	6.7	0.0125*	12.6	3.5	0.0382*	13.2
AGRNg	21.0	0.0000*	70.9	7.3	0.0004*	32.6	13.4	0.0006*	22.3	3.6	0.0364*	13.2
AGRh	150.6	0.0000*	94.6	17.2	0.0000*	53.4	41.6	0.0000*	46.9	1.8	0.1735	7.3
AGRA	21.0	0.0000*	70.3	6.9	0.0006*	31.5	9.7	0.0031*	17.1	5.3	0.0082*	18.9
RGR	415.6	0.0000*	98.0	322.6	0.0000*	89.0	291.6	0.0000*	89.0	4.8	0.0133*	17.1
RGRA	871.2	0.0000*	99.0	512.8	0.0000*	90.0	427.5	0.0000*	89.5	4.8	0.0128*	17.3
RGRWL	95.3	0.0000*	91.7	16.3	0.0000*	52.0	24.8	0.0000*	34.5	5.2	0.0089*	18.6
RGRNL	300.4	0.0000*	97.2	282.9	0.0000*	87.2	306.7	0.0000*	87.2	4.7	0.0138*	17.0
RGRh	194.3	0.0000*	95.8	130.8	0.0000*	85.7	124.2	0.0000*	85.6	5.4	0.0081*	18.9
Dynamic allometric morphoparameters												
NAR1	18.2	0.0000*	67.8	5.3	0.0033*	26.0	11.0	0.0018*	18.9	2.1	0.1289	8.5
NAR2	13.4	0.0000*	63.2	4.5	0.0072*	24.1	9.5	0.0033*	16.4	2.1	0.1334	8.0
LAR1	23.4	0.0000*	73.2	6.8	0.0007*	31.1	9.7	0.0032*	17.0	5.2	0.0090*	18.5
LAR2	13.4	0.0000*	68.2	26.2	0.0000*	63.6	4.5	0.0400*	8.7	5.5	0.0070*	19.4
LAR3	10.3	0.0000*	54.4	12.3	0.0000*	45.0	1.8	0.1820	5.3	5.0	0.0112*	17.7

[1]The mark * identifies morphoparameters, for which the factor showed statistically significant influence ($p < 0.05$)

The highest values of all indicators of relative growth rate were registered in the *Numphoides peltata - Ceratophyllum demersum* community and the lowest values (in most cases) were registered in the *Nuphar lutea + Potamogeton natans* community. The rates of relative growth rate in *Nymphaea candida* are low in variation. All the ecological-cenotic factors studied showed a statistically significant influence on the value of all indicators of relative growth rate. However, the influence of the factors was different: water column 91.7-99.0%, total plant cover 52.0-90.0%, bottom sediments 34.5-89.5%, water transparency 17.0-18.9%. The most significant influence on this group indicators has a water column.

Dynamic allometric morphoparameters. The highest values of net-assimilation (NAR1) are found in the coenopopulations of the *Nuphar lutea -Ceratophyllum demersum* and *Nymphaea candida - Potamogeton lucens* (0.04±0.007 g/cm^2/day). The NAR1 value is 0.02±0.001 g/cm^2/day in the *Nuphar lutea + Potamogeton natans* community. Specimens of the coenopopulations of the *Nymphaea candida subpurum*, *Nuphar lutea subpurum* and *Numphoides peltata - Ceratophyllum demersum* have the lowest values of this morphoparameter (0.01±0.001 g/cm^2/day).

The highest values of net-assimilation (NAR2) were observed in the *Nymphaea candida - Potamogeton lucens* and *Nuphar lutea - Ceratophyllum demersum* (0.009±0.0001 g/cm^2/day) communities, and the smallest were observed in *Numphoides peltata - Ceratophyllum demersum* (0.002±0.0001 g/cm^2/day). In the *Nuphar lutea subpurum, Nymphaea candida subpurum* and *Nuphar lutea + Potamogeton natans* communities, the values of NAR2 range from 0.004±0.0003 g/cm^2/day to 0.006±0.0001 g/cm^2/day.

The rates of leaf area formation productivity (LAR1, LAR2, LAR3) vary over a wider range than net-assimilation. The highest LAR1 value is in individuals of the cenopopulation of the *Nuphar lutea - Ceratophyllum demersum* community (166.3±23.28 cm^2/g/day), the lowest is in the *Numphoides peltata - Ceratophyllum demersum* (9.8±2.26 cm^2/g/day) which is 17.0 times less. The highest values of LAR2 and LAR3 are in individuals of the cenopopulation of the *Nymphaea candida subpurum* community (0.70±0.042 cm^2/g/day and 5.4±0.29 cm^2/g/day, respectively), the smallest values are in *Nuphar lutea + Potamogeton natans* (0.21±0.006 cm^2/g/day and 2.8±0.07 cm^2/g/day, respectively), which is 3.3 and 1.9 times smaller, consequently.

The factors of the water column and total plant cover showed a statistically significant effect on all dynamic allometric morphoparameters, affecting the levels of 54.4-73.2% and 24.1-63.6%, respectively. The bottom sediments and the water transparency did not have a statistically significant effect, respectively, on one (impact force 8.7-18.9%) and two morphoparameters (impact force 17.7-19.4%). The leading factor that determines the patterns of variation of dynamic allometric morphoparameters in *Nymphaea candida* is the water column. Dynamic morphoparameters in *Nymphaea candida* have a low level of variation. For most morphoparameters, the variance and standard deviation values are smaller than one.

The data obtained from the study of the growth characteristics of *Nymphaea candida* are agreement with the growth information of other aquatic plant species: *Trapa natans* and *Potamogeton natans* (Skliar, 2017; Skliar & Skliar, 2017). In particular, they argue that dynamic morphological parameters are important indicators of the macrophyte populations condition. According to their values, habitats and (or) reservoirs, is possible to differentiate by the degree of favorable for the functioning of populations of these species and, on this basis, to develop approaches for the protection of aquatic plants and, if necessary, the principles of their sustainable, inexhaustible economic use.

CONCLUSIONS

In *Nymphaea candida*, the rate of growth processes varies substantially depending on the growing conditions. An analysis of the effects of the leading ecological-cenotic factors showed that in the study area, the water column and the total plant cover of phytocenosis statistically significantly influence all the dynamic (metric and allometric) morphological parameters of *Nymphaea candida* plants. The influence of these two factors is 54.4-99.0% and 23.6-90.0%, respectively.

The bottom sediment characteristics did not show a statistically significant effect on only one dynamic allometric morphoparameter (LAR3), and the influence of this factor on all other dimensional features of *Nymphaea candida* plants varied within 8.7-89.5%. Water transparency did not show a statistically significant effect on three morphoparameters (one metric - AGRH, two allometric - NAR1, NAR2) with the influence of this factor on all other features at the level of 13.2-19.4%. Thus, taking into account the set of indicators characterizing the effect on the growth of *Nymphaea candida* plants, the studied ecofactors form the following series (in order of increasing magnitude and significance): water transparency → bottom sediments → total plant cover →water column.

In the study region in plants of *Nymphaea candida* the highest values of most (58.8%) dynamic morphoparameters are registered in the community *Nymphaea candida - Potamogeton lucens*. The smallest values of most morphological parameters (64.7%) are registered in the community *Numphoides peltata - Ceratophyllum demersum*.

At the same time, plants of *the Numphoides peltata - Ceratophyllum demersum* community exhibit the highest magnitudes of almost all (four out of five) indicators of relative growth rate.

The fastest growth of *Nymphaea candida* plants is in the areas where there is no flow, the water column varies within 50-100 cm, the water transparency reaches the bottom, the silt bottom sediments are represented, and the total plant cover of phytocenosis is 60-85%. The projective coverage of *Nymphaea candida* can range from 10% to 40%. Such habitat conditions are optimal to ensure the continued existence of populations of this species as a whole.

Accordingly, the introduction of active measures for the protection of populations of *Nymphaea candida* in certain reservoirs (their areas) should be oriented towards the achievement of such a complex of ecological-cenotic characteristics.

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WHAT ATTRACTS TOURISTS IN RURAL AREAS? AN ANALYSIS OF THE KEY ATTRIBUTES OF AGRITOURIST DESTINATIONS THAT MAY INFLUENCE THEIR CHOICE

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Abstract

Agritourism represents a fast-growing industry in Romania, with an average growth rate of tourist arrivals four times higher than the sectorial one. However, there are strong regional differences in the flow of tourists attracted, whereas the ability of the specific destinations to attract tourism flows, given their endogenous characteristics, are insufficiently studied. The paper analyses tourist flows between 2007-2016 from the supply-side of tourist products perspective taking into consideration the particularities of tourism destination (natural resources, infrastructure, socio-demographic aspects etc.). The dependent variable in this study is the number of new overnight stays, as a proxy for the attractiveness of the region. The results of the binary logistic confirm the importance of tourism infrastructure variables, as well as natural environment and socio-demographic aspects in determining domestic tourism flows. Thus, the demographic elements such as population density, young women population rate, young working population rate can give an important comparative advantage to a tourism destination. Moreover, the study highlights the presence of other relevant determinants such as the area occupied with meadows and ponds, as elements of the natural environment.

Key words*: agritourism, logistic regression, attractiveness, natural environment, socio-demographic, Romania.*

INTRODUCTION

The tourism demand has seen solid growth in the last decades due to the economic, social, political and technological changes. However, it sees both fluctuations, due to the volatility of the various determinants (Song, 2019), as well as strong regional differences in the flow of tourists attracted. The regions are competing to attract these resources and it is obvious that they are interested in analyzing the influencing factors of the tourist flow's dynamic and size. Understanding these drivers allows launching appropriate strategies, transmission of promotional messages that will lead to attracting as many tourists to specific destinations. An impressive scientific literature is dedicated to these topics, being unanimously acknowledged that the rapid expansion of international tourism is mainly due to the high rates of income growth in developed and emerging countries, the reduction of working time and the substantial reduction of transport costs. There is a large number of scientific articles that deal with issues related to the relationships between tourism demand (considered either from the perspective of tourist expenses and receipts, tourist arrivals or tourist overnights) and the variables that determine the flow of tourists (Crouch, 1994; Witt et al., 1995; Lim, 1997; 1999; Morley, 1998; Sinclair, 1998; Cho, 2001; Song et al., 2010; Dogru et al., 2017; Santamaria and Filis, 2019; McKercher and Mak, 2019).

Most of the conducted studies focus primarily on the economic variables that affect the tourist demand, considering the incomes and prices as significant factors that influence the decision to travel, validating the hypothesis that the tourist demand is positively influenced by revenues and negatively influenced by prices (Lim, 1999; Fuleky, 2014; Hanafiah and Harun 2010; Zhang and Jensen, 2007). A number of non-economic variables are considered, although in a small number of studies: population density, cultural heritage, political stability, poverty level (Lyons, 2009), tourism infrastructure (Seetanah, 2011), advertising expenses (Song and Witt, 2010), changes in tourists' tastes (Lim, 2004), natural and cultural assets (Romão et al., 2017), autochthonous breeds (Zrakić et al., 2018), distance (McKercher and Mak, 2019).

The importance of these variables increases if we consider that domestic demand is less sensitive to changes in income and prices than international one (Seddighi and Shearing, 1997; Garin-Munoz, 2009), and this is important for countries where domestic tourism dominates. However, the vast majority of studies regarding the analysis of the tourism demand dynamic have focused on the international flow by using aggregated data at national level (Song, 2019). This is also the case of Romania where, although there are relatively few studies on the modeling of tourism demand and on the dynamics of the tourist flow, their vast majority focuses on the international flow. Economic variables, such as GDP per inhabitant, international trade flows, population, distance and prices are generally used (Surugiu, 2011).

However, the analyzed determinant factors represent predominantly exogenous variables over which the tourist destinations, local or national, have a reduced power of influence and control (Massidda, 2012). Aspects regarding the attractiveness of different territories, the ability of certain destinations to attract important flows given their endogenous characteristics (natural environment, infrastructure, economic vitality, socio-demographic aspects, etc.) are insufficiently or not at all studied. Song et al. (2010), Zhang and Jensen (2007) and Li et al. (2005) find that the impact of tourism supply in the tourism demand literature is addressed insufficiently. Although it is recognized that factors such as distance, tourist services and infrastructure, natural and cultural resources can strongly influence tourists' choices (Massidda, 2012).

The purpose of this paper is to test whether the attributes of tourist destinations are important in order to explain the attractiveness of agritourism, measured in the number of overnight stays at rural community level. To analyze the characteristics associated with the attractiveness of agritourism destinations, the logistic regression model on a cross section covering all (2858) rural localities in Romania was used. The territorial level of analysis is exactly the final destination for agritourism consumption, the rural community (LAU 2).[1]

The present study contributes to the enrichment of the existing literature from several perspectives. First, as regards the general contribution to the academic literature, the study extends the research on the determinants of demand within a particular form of tourism, agritourism, with a special dynamic in recent years. Secondly, it is one of the few studies that takes into account the supply-side characteristics, such as: general natural characteristics (land fund structure - cover land -, the share of forests, the built space, the communication paths and the uncultivated land); socio-demographic characteristics (age and sex structure of the population, density and population growth dynamics); distance to the nearest city of 15000 inhabitants and to the county capital. It thus contributes to the understanding of the relations between the territorial resources and the dynamics and attractiveness of tourism at local and regional level. Or, in other words, it is trying to answer the question of why certain regions, localities are more successful in attracting and maintaining a high flow of tourists and others not. Do they differ by a certain mix of features and resources of their offer? The tourism products, as a combination of tourist resources and services, are heterogeneous and differentiated, each destination thus having the capacity to offer unique experiences. Are there, however, certain common attributes of the regions that manage to attract, year after year, increasing flows of tourists compared to the areas excluded from this economic activity?

Third, the analysis is performed at LAU 2 level, representative level to capture the impact of this particular form of tourism, agritourism, and the study covers all rural communities of Romania. From this point of

[1]Local Administrative Units level 2 (formerly NUTS level 5) consisted of municipalities or equivalent units in the EU Member States

view, this analysis is, to our knowledge, the first performed in Romania and among the few in Europe, at such a level of details.
The work is organized as follows. The following section analyzes the recent trends that characterize the tourist flows in Romania.

MATERIALS AND METHODS

Models and trends in domestic tourism flows in Romania

Tourism is gaining in importance in the recent decades in Romania, both in terms of contribution to the economic growth, employment but also as a driving force of the international trade.
The known sensitivity of this economic sector to the economic and social stimuli has determined an increase of the demand as well as of the tourist offer in Romania between 2000 and 2016, differentiated by forms of tourism and by regions (Tenie et al., 2018). Thus, the average annual growth rate of total tourist arrivals during 2007-2016 was 6.28%. However, a level of 4 times higher is registered for arrivals within the agritourist guest houses (Table 1).

Table 1. Tourists arrivals, by types of tourist accommodation structures, 2007-2017

Types of tourist structures	Years		Average annual growth rate
	2007	2017	2016/2007
	UM		
	No. of persons	No. of persons	%
Total	6,971,925	12,143,346	6.28
Tourist guest houses	451,640	1,157,665	9.64
Agritourist guest houses	288,508	1,004,400	23.71

Source: own calculations according to the National Institute of Statistics (INS)

However, there are big differences at regional level in terms of the ability to attract tourist flows. The Central Region is the main destination, with the highest number of overnight stays in agro-touristic guest houses (40%) during 2007-2016.
Together with the North-West and North-East Regions, they total more than 75% of the total number of overnight stays in the agritourist guest houses during the analyzed period. The South-East and South regions lose their attractiveness during this period, with a decrease of the share held in the total number of overnight stays (Table 2 and Figure 1).

Table 2. The share of tourist nights in the agritourist guest houses in Romania between 2000-2016 (%)

Region/ Years	**2007**	**2010**	**2016**
TOTAL	**100**	**100**	**100**
North-West Region	24.06	17.13	17.18
Central Region	32.33	33.66	39.19
North-East Region	19.82	22.29	18.33
South-East Region	6.72	7.48	3.42
South Region	9.09	8.12	7.78
Bucharest - Ilfov Region	0.87	0.25	0.05
South- West Region	2.88	5.16	6.78
West Region	4.24	5.91	7.27

Source: own calculations according to the National Institute of Statistics (INS)

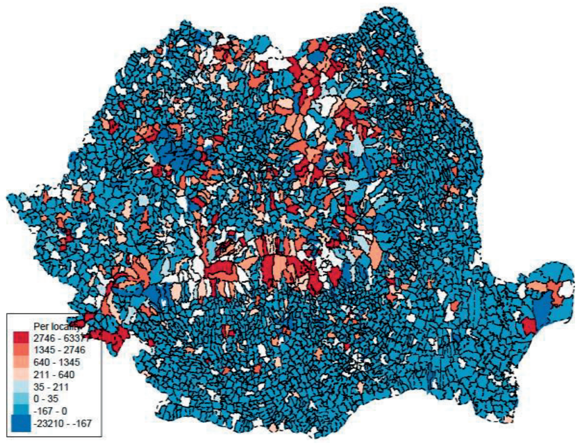

Figure 1. New overnight stays in the period 2007-2016 within the agritourist pensions, by localities
Source: Spmap STATA processing according to the INS data

An important role in increasing the accommodation capacity and the quality of services is played by the investment made during this period.
During 2007 - 2016 the investments in the extension and modernization of the agritourist accommodation capacity, realized by the National Program for Agriculture and Rural Development (measure 313) varies from 0 to 2,132,324 euro/locality.
It is worth noting their concentration in the Central-North-Western Regions (Figures 2 and 3).

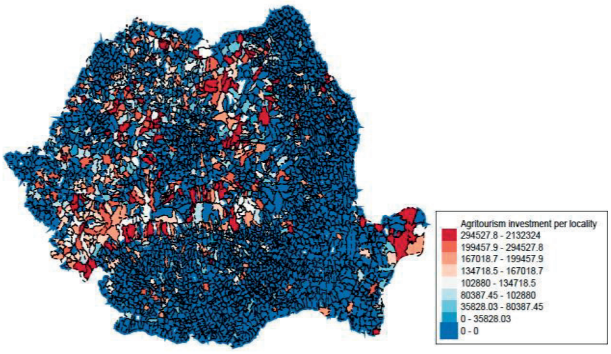

Figure 2. Investments made by the PNADR 2007-2013 (measure 313), by localities, Euro
Source: Spmap STATA processing according to the AFIR data

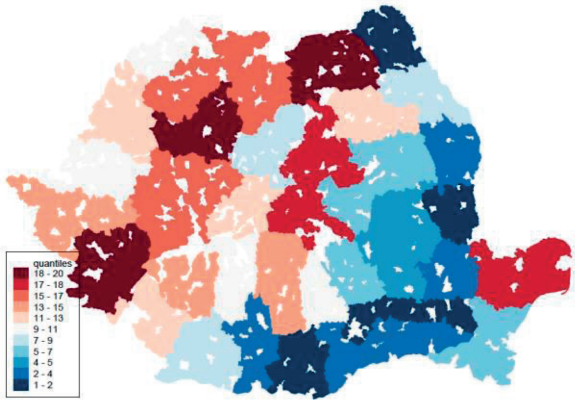

Figure 3. Distribution of investments made by the PNADR 2007-2013 (measure 313), by counties, quantiles
Source: Spmap STATA processing according to the AFIR data

In conclusion, the recent dynamics of agritourism flows show an increase of tourist flows concentration in certain tourist regions. Therefore, it seems natural that the attention should be directed towards knowing and understanding the role of the main determinants of the size and trajectory of tourist flows. This information is useful for implementing measures that could lead to increased regional attractiveness. This paper aims to investigate the importance of the attributes of the tourist destinations in establishing the tourist flows to the agritourist guest houses located at the LAU level. For this purpose, both economic variables and non-economic variables proposed by the scientific literature were considered, such as: distance, tourist infrastructure, tourist services, tourist investments (Massidda, 2012). A series of indicators related to the natural resources and demographic dynamic and structure was also taken into consideration.

Description of data and research method

The set of attributes and indicators was selected so as to identify the important characteristics of the destinations for rural tourism and agritourism, the limitations being given by the availability of the data and the relevant proxies for offer characterization. Data sources were provided by National Institute of Statistics (INS), Ministry of Agriculture and Rural Development (MADR), Agency for Financing Rural Investments (AFIR).

Most of the studies aimed at analyzing the evolution of the tourist services demand, published in recent years, have used longitudinal data or time series (a destination analyzed over a period of time), with very few of those using cross-sectional data analysis, from several tourist destinations; even fewer are the analyzes that were based on the use of pooled data/panel study: time series for a number of tourist destinations to estimate or evaluate the impact on tourism demand both in time and at cross-sectional level. Unlike the time series that try to capture demand changes over a period, cross-sectional data aims to increase the understanding of the determinants of tourism demand that orient the tourist flow to one or another of the tourist destinations.

The present study is based on the grouping of statistical information so as to reproduce the image of the evolution of the tourist demand between 2007 and 2016, but also at a cross-sectional level, for all rural localities in Romania. This allows us to discern how the tourist demand has evolved over time in each transversal unit, incorporating information from both time series and cross-sectional data.

The attractiveness of the region for the tourist demand - the dependent variable in this study - is measured in the number of new overnight stays, as a proxy for the attractiveness of the region for the tourist demand between 2007-2016:

$$Y_i \; 2007\text{-}2016 = (\text{Overnights } 2008 - \text{Overnights } 2007) + (\text{Overnights } 2009 - \text{Overnights } 2008) + (\text{Overnights } 2016 - \text{Overnights } 2015) \quad (1)$$

where:

Y_i = number of new overnight stays between 2007 and 2016 at the locality i level.

The explanatory variables were selected according to their ability to best describe the tourism offer, taking into account the availability of information at this territorial level (LAU2). The following were included:

1) Variables that describe the general natural resources, such as the structure of the land per inhabitant (total land, arable area, pastures, ponds), as a proxy for territorial diversity. Indicators were calculated per inhabitant (data on natural resources were divided by the number of inhabitants in each commune/village) to take into account the possible size effects.

(2) Socio-economic variables: population growth, young population rate and female population rate, were considered as a proxy for the dynamism and economic diversity of the locality, capable of attracting and maintaining the population, especially the young and female population. The number of new jobs created in the primary, secondary and tertiary sectors was also analyzed.

3) Territorial and tourism infrastructure variables. Unlike other economic activities, in tourism, both tourism production and consumption coincide in time and space and take place in tourist destinations. Tourist destinations need a certain level of infrastructure to be attractive enough and to allow the tourist circulation. In addition to the standard variables for infrastructure (accommodation capacity existing at the locality level at the beginning of the period), the data on the "newly created assets" and the investments made during the period 2007 - 2016 in agritourist guest houses through PNDR were considered important, being generative both of knowledge and of reputation.

4) Other attributes of the offer include the distance to the nearest city of 15,000 inhabitants to evaluate the influence of urban proximity on the dynamics of agritourism flows (Tables 3 and 4).

We set out to explain the probability of the dependent variable (the number of new overnight stays per locality in the period 2007-2016) based on a set of independent variables using the Logistic regression model, the maximum likelihood (ML) method.

Table 3. Database Description

Variable category	Description	Territorial level	Time	Source
Natural resources variables				
Natural resources/ Land use	Utilized agricultural area (UAA, ha) Area covered with arable land/inhabitant Area covered with peadows/inhabitant Area covered with pastures/inhabitant Area covered with ponds/inhabitant	LAU2	2007 2010 2016	INS MADR
Socio economics variables				
Demographics	Number of inhabitants per SKm Young population rate 2016 Young women population rate 2016	LAU2	2009–2016	INS
Territorial/Infrastructure indicators				
Tourist capacities	Tourist capacities (no. places) Agritourist capacities (no. places)	LAU2	2007–2016	INS
Agritourist investment	Payments for agritourism investment approved projects - Rural development Program, Measure 313	LAU2	2007–2014	AFIR /
Distances to urban areas	Kilometers from the community to the next small town (15,000 inhabitants)	LAU2	2016	GIS–own calculations
Touristic flux				
Tourist arrivals	The number of tourists accommodated in agritourist boarding houses	LAU2	2007–2016	INS
Tourist Overnights	The number of overnight stays in agritourist boarding houses	LAU2	2007–2016	INS

INS: Romanian National Institute of Statistics
MADR: Ministry of Agriculture and Rural Development
AFIR: Agency for Financing the Rural Investments

We set out to explain the probability of the dependent variable (the number of new overnight stays per locality in the period 2007-2016) based on a set of independent variables using the Logistic regression model, the maximum likelihood (ML) method. The regression equation obtained allows an estimation of the probability of producing the analyzed event according to the values of the independent variables.

Logistic regression is a solid model used to estimate the probability of the event, validated in many researches in the field of tourism, and in social sciences in general (Chu, 2014).

Table 4. Descriptive statistics of the variables used in the analysis

Variable	Description	Number Observations	Mean	Std. Dev	Min	Max
Arable_land~H	Area covered with arable land/inhabitant	2,858	1.023297	1.085447	0	23.7717
Pastures_H	Area covered with pastures/inhabitant	2,858	.417106	.6456871	0	10.74766
Meadows_H	Area covered with meadows/inhabitant	2,858	1898746	.3475924	0	5.453532
Ponds_H	Area covered with ponds/inhabitant	2,858	.1198509	1.431843	0	56.56625
PopChange	Change in the number of residents over the period (2010-2015);	2,858	-.0209623	.1152969	-.4566742	2.66863
Pop_Density	Number of inhabitants per SKm	2,858	61.69491	49.1834	1.505547	595.5869
YoungWom~_16	Young women population rate 2016	2,858	.4685513	.0185661	.3409091	.5659599
YoungWorkRatio _16	Young working population rate 2016	2,858	.6343499	.0405445	.4147287	.8186238
CapacityA_~7	Agritourist capacity in 2007 (no. places)	2,858	5.140559	36.73644	0	1057
w_CapacityA_~7	Agrotourist capacity in 2007 quantiles		4.93177	6.459039	1	20
w_OtherEst~c	Other Tourist capacities except agritourism (quantiles)	2,858	3.398181	4.722501	1	20
NewAgritou~c	New agritourist capacity 2008-2016 (no. places)	2,858	7.058042	44.20937	-483	1272
w_NewAgrit~c	New agritourist capacity 2008-2016 (quantiles)	2,858	4.009091	6.665541	1	20
RPMeasur~313	Payments for agritourism investment approved projects - Rural development Program, Measure 313 Euro/locality	2,858	46957.63	125961.6	0	2132324
NewOvernig~A	New agritourist overnights 2007 -2016	2,858	327.4329	2356.421	-23210	63377
Dist_~1500	Kilometers from the community to the next small town (15,000 inhabitants)	2,859	20.00197	11.16418	0	78.77375

Source: Own processing in STATA

In the field of tourism, the logistic estimation model has been used to analyze the determinants of business travelers loyalty (Tsaur et al., 2002), the determinants of innovation in services and marketing (Divisekera, 2018), of the competitiveness of tourism destination in developing countries (Goffi, 2019).

In the current study, the dependent variable, the number of new overnights per locality in the period 2007-2016, was coded 0/1, becoming thus dichotomous, in order to mark the belonging to the two categories - presence/absence of overnights-, for each rural locality: (a) with value 1 for the localities with more overnight stays than 0 and (b) with the value 0 for the localities with the number of nights less than or equal to 0 during the analyzed period.

The general model is:

$$\ln\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots \beta_i X_i \quad (2)$$

where:

- p = P (Y = 1), p is the probability of realizing the value Y = 1 for the given values X1, X2, X3 ... Xi. In other words, the probability that the values of the independent variables xi are associated with the production of the event Y = 1.

$\left(\frac{p}{1-p}\right)$ represents the odds ratio, that is the ratio between the probability that the values of the independent variables are associated with "the presence of the event, the success" and the probability of being associated with "the absence of the event, failure". The logistic regression model is directly associated with this notion of odds ratio.

$$\text{Odd} = \frac{p}{1-p} = \frac{\text{Probability of event occurring}}{\text{Probability of event not occurring}}$$

- β1 ... βi are the regression coefficients calculated using the statistical program STATA, and express the contribution of factor xi to the explanation of the probability in the form of the chance ratio of the event production Y = 1. These are in fact natural logarithms of the chance ratio ("odd ratio") for each variable. The logit transformation is necessary to design the probability p from the interval (0.1) to the interval (- ∞, + ∞), and for this case it is possible to estimate the parameters).

- β0 is constant, representing the natural logarithm of the odds ratio ("odd ratio") for observations that have zero values for all independent variables.

If $(p/(1-p)) > 1$, this means that $p > 0.5$, then the observation is more likely to belong to the group characterized by $Y = 1$, ie logit > 0.

Logistic regression predicts a binary effect variable Y (with or without overnight stays, respectively values 1 and 0), based on one or more independent variables X1, X2, X3 ... Xi. Instead of working with probabilities (between 0 and 1), the logistic regression uses the natural logarithm of the odds rate, which can take any value, positive or negative.

RESULTS AND DISCUSSIONS

The results of the binary logistic regression are summarized in Table 5. The applied model is statistically significant (chi-square = 1275.15, p = 0.00). The verification of the model was done by calculating the sensitivity and specificity (Table 6), with a percentage of 91.88% correctness of the classifications, the dependent variable being correctly classified in 82.26 and 92.07% of the cases respectively.

The logistic model shows that the probability of occurrence of the event is a function of the multiplicative effects of the different variables.

The coefficients of the logistic function (i.e., how much the probability logarithm of the dependent variable changes with a unit change of the independent variable) are more difficult to interpret. Thus, we found it useful to obtain the odds ratio, which shows us the probability of the independent variables to be associated with the presence of the tourists stays or with their absence.

Table 5. Logistic regression model results (coefficients and Odds Ratio)

Variable	Coef.		Odds Ratio	
NewAgritour~c	.1258629***	.0071294	1.134127***	.0080856
OtherEstabl~s	.0132549***	.0029361	1.013343***	.0029753
CapacityA_2~7	.0337015***	.0064035	1.034276***	.006623
RPMeasure~313	2.38e-06***	7.06e-07	1.000002***	7.06e-07
Arable_land_H	-.6504554***	.1331587	.5218081***	.0694833
Pastures_H	-.3875549*	.1866717	.6787144*	.1266968
Meadows_H	.9925917***	.3065068	2.698218***	.8270221
ponds_2010_H	.1707834**	.0564108	1.186234**	.0669164
Pop_Density	-.5028588**	.1748799	.6047992**	.1057672
YoungWorkR~16	4.447071*	1.912718	85.37654*	163.3012
YoungWomenR~6	11.41322*	4.710073	90510.26*	426309.9
Distanta~1500	-.0323769***	.0075431	.9681416***	.0073028
	Number of obs = 2,858 LR chi2(24) = 1263.98 Prob > chi2 = 0.0000 Pseudo R2 = 0.4792 Log likelihood = -686.88326 Stars denote p-values as follows: *p < 0.05; **p < 0.01; ***p < 0.001.			

Source: Own processing in STATA, using logit and logistic function

The obtained results reveal:

1. Statistically relevant and positive relationship, with the following variables: The agritourist capacity in 2007; The tourist capacity existing in other forms of accommodation than agritourism in 2007; The investments made between 2007-2016 by measure 313; New tourist capacity created between 2008-2016 (number of accommodation places); The area occupied by meadows per inhabitant; The area occupied by lakes per inhabitant; Rate of young working population, Rate of young female population.
2. Statistically and negatively relevant relationship, with the variables: Arable area per inhabitant; Area occupied by pastures per inhabitant; Population density; Distance from cities with less than 15000 inhabitants.

Table 6. Clasification table: Logistic model for LogOvernightA

Classified	-------- True --------		Total
	D	~D	
+	310	48	358
-	186	2314	2500
Total	496	2362	2858
Classified + if predicted Pr(D) >= .5 True D defined as LogOvernightA != 0			
Sensitivity		Pr(+| D)	62.50%
Specificity		Pr(-|~D)	97.97%
Positive predictive value		Pr(D| +)	86.59%
Negative predictive value		Pr(~D| -)	92.56%
False + rate for true ~D		Pr(+|~D)	2.03%
False - rate for true D		Pr(-| D)	37.50%
False + rate for classified +		Pr(~D| +)	13.41%
False - rate for classified -		Pr(D| -)	7.44%
Correctly classified			91.81%

Source: Own processing in STATA

The existing and newly created tourist capacity is a statistically significant factor that positively affect the probability of new overnight stays at the level of the specific locality. The positive relation with other forms of accommodation, different from agritourism, can be interpreted from the perspective of the tourist vocation, of the reputation of the respective region, rich not only in agritourist resources but also in a wide range of other natural and anthropic resources. Given the financiers standards, the new existing materials and technologies, then the new tourist capacity created during 2008-2016 can be considered as a proxy for a high quality of the services and the accommodation infrastructure.

The area occupied with meadows and ponds, as elements of the natural environment, have statistical significance in determining the probability of occurrence of additional overnight stays. These relationships can be explained by the fact that these categories of land structure, besides the fact that they are elements of the landscape of great value for biodiversity conservation, favoring outdoor recreational activities, are also labor extensive and allow the active population to engage in alternative activities, i.e. tourism. Unlike these variables, arable area per inhabitant and pastures area per inhabitant, although statistically significant, they negatively affect the probability of additional overnight stays in the respective localities. These relationships could be explained by the fact that the technologies applied to these lands are much more labor-intensive or technology-intensive (with effects of scale economy). The high share of areas occupied by pastures is generally connected with the existence of larger livestock (unfortunately unavailable data to be taken into account at community level) which again may involve limited time resources to be dedicated to the development of other complementary activities. However, where the migration did not involve the young population, and especially young women population it is more likely to have the appearance of additional overnights. In fact, the connection between the female population and its involvement in tourism activities is already known (Obadic & Maric, 2009). The findings seem to suggest a negative statistically significant relationship between the population density and probability of new agritourist overnights. This could mean that agritourism is perceived as an escape from the large urban agglomerations, associated therefore to less crowded areas. In this context, the statistically significant but negative relation of the distance to the small urban centers deserves a nuanced interpretation. On the one hand, in a well balanced regionaly system, these small and medium towns acts as growth nuclei, to ensure the urban-rural connection. On the other hand, in Romania, many of these small settlements lack strategies for promoting the town image (Bănică A., Camară G., 2011). Monoindustrial centers before 1989 are fighting for diversification of the activity and economic revitalization, not yet being able to coagulate important flows of tourists to benefit the peripheral rural areas.

CONCLUSIONS

Over the last decades, agritourism proved to be a fast growing industry in Romania, with an average growth rate of tourist arrivals four times higher than the sectorial one. In the current tourism literature, tourism flows are typically explained by the demand-side variables, mainly measuring the tourism income and price elasticity, ignoring the particularities of the tourist products. However, there strong regional differences in the flow of tourists attracted, whereas the ability of the specific destinations to attract tourism flows given their endogenous characteristics are insufficiently or not studied at all. This paper analyses tourist flows from a different perspective: the supply-side of tourist products taking into consideration the particularities of tourism destination (natural environment, infrastructure, socio-demographic aspects, distances to urban areas etc). The dependent variable in this study is measured in the number of additional overnight stays, as a proxy for the attractiveness of the region for the tourist demand between 2007-2016. The Logistic regression model is used, as a solid model used to estimate the probability of event production, validated in many researches in the field of tourism, and in social sciences in general. This model aimed at providing information on the attributes of the tourism supply that influence the tourist demand/the additional overnight stays within the rural communities.

The results of the binary logistic model are rich in powerful insights. They confirm the importance of economic variables in determining domestic tourism flows, in accordance with the previous literature. Thus, there is statistically relevant and positive relationship between the tourism flows and the tourism investment, the newly created tourist capacity as well as the existing capacity at the beginning of the analyzed period. Moreover, the study highlights the presence of other relevant determinants such as other form of tourist accommodation, except for agritourism accommodation, proving the importance of the tourist vocation of the specific area.

At the same time, statistically significant coefficients, prove that the demographic elements of tourism supply such as population density, young women population rate, young working population rate can give an important comparative advantage to a tourism destination. The area occupied with meadows and ponds, as elements of the natural environment, have statistical significance explaining the probability of occurrence of additional overnight stays.

Elements of the tourist attractiveness that were not considered due to the lack of data request further investigation, to include: activities that can be carried out locally, considered as an important factor of the experiential economy; the attitude of the community/locals towards tourism. Interesting and useful would also be for further research a mapping of the tourist attractions and their proximity to urban and rural settlements.

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THE BEHAVIOR OF FOUR WINTER WHEAT GENOTYPES UNDER DIFFERENT RATES OF NITROGEN FERTILIZER

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Abstract

In agriculture water and nitrogen are the main factors that influence crop production in all regions. Interactions between water and nitrogen fertilization are complicated. The present study investigated the influence of nitrogen fertilization rate and rainfall distribution on plant height, grain yield and grain protein content of four winter wheat varieties in a three years field experiment in Turda, Romania. To establish the relationships between nitrogen fertilization rate, rainfall distribution and grain yield the follow parameters was determined: the rain use efficiencies (RUE), nitrogen agronomic efficiency (NAE) and nitrogen use efficiency (NUE). Andrada and Codru had a significant higher RUE which means that this genotypes produced higher rate of grain yield per unit rainfall than Arieşan and Taisa. The highest NAE was recorded by Taisa which means that this cultivar has the highest ability to increase yield in response to the rate of nitrogen fertilizer applied. Andrada and Codru had the highest value of NUE, which means that these cultivars have the ability to use more efficiently the nitrogen to obtain a higher yield.

Key words: *NAE, NUE, RUE, winter wheat.*

INTRODUCTION

Wheat is one of the most widely grown cereal crops in the world due to its importance for various food and feed products, and its wide genetic adaptability to varying environmental conditions such as temperature, moisture and light (Pavuluri et al., 2015; Muntean et al., 2014).
Although world food needs are increasing, climate changes, such as rise in temperature and decline in rainfall may negatively affect crop yields in some major production regions of the world (Kadar et al., 2019). Wheat genotypes that efficiently capture and convert available soil nitrogen into harvested grain protein are the key to sustainably meeting the rising global demand for grain protein (Guttieri et al., 2017).
In agriculture water and nitrogen are two primary factors in crop production in all regions. Nitrogen fertilization is a common practice to increase food production, but its performance depends on soil water status (Halvorson et al., 2004; Turner, 2004; Turner and Asseng, 2005). Typically rain-fed farming system are characterized by the absence of irrigation, here the main source of soil water supply for crop growth is precipitation. Annual and seasonal precipitation can influence crop response to nitrogen fertilization, accounting for major variations in yield, water use efficiency, and nitrogen use efficiency (Guo et al., 2012). Interactions between water and nitrogen fertilization are complicated and may cause either positive or negative effects on crop growth (Li et al., 2009).
Rain Use Efficiency (RUE) according to Gwenzi et al. (2008) is a parameter defined as grain yield divided by total seasonal rainfall, is an indicator that expresses grain yield per mm of rainfall. It has been used to analyze the variability of vegetation production (Dardel et al., 2014).
Nitrogen use efficiency (NUE) according to Moll et al. (1982), is a parameter that expresses grain dry matter yield per unit available nitrogen. NUE is the net result of N capture (uptake efficiency) and N conversion (utilization efficiency) (Guttieri et al., 2017). Genetic associations between grain yield and NUE, and variation in NUE among genotypes

have been widely recognize in various studies (Foulkes et al., 2009).

Nitrogen agronomic efficiency (NAE), is a parameter that represent the ability of the plant to increase yield in response to the amount of nitrogen rate applied (Delogu et al., 1998).

This study investigated the relationships between nitrogen fertilization rate, rainfall distribution and grain yield based on a three years field experiment with three nitrogen fertilization rates in Turda, Romania. Its objectives were to: study the impact of seasonal precipitation on grain yield and plant height, to determine the rain use efficiencies (RUE), nitrogen agronomic efficiency (NAE) and nitrogen use efficiency (NUE) of four winter wheat varieties under different nitrogen rates and to determine the influence of nitrogen rates on plant height, yield and grain protein content.

MATERIALS AND METHODS

The experiments have been carried out at the Agricultural Research and Development Station Turda (46°35' N latitude and 23°47' E longitude, 345-493 m above Adriatic Sea), which is located in the Transylvanian Plain, Romania. The field experiment was established on a clay Chernozem soil, typical for this region. The agrochemical parameters for this type of soil are: the soil reaction is neutral (pH 6.9-7.1) and the humus content is between 3.56-3.92%. The soil is rich in nitrogen (0.183-0.196%), and potassium content (249 ppm), and poor in mobile phosphorus (15 ppm).

The experimental design consists in subdivided plots in a three factorial experimental system. The experimental factors were:

- Genotype (G);
- Nitrogen fertilization (N);
- Season (S).

For this study were used four winter wheat genotypes created at ARDS Turda: Arieşan, Andrada, Codru, Taisa. Nitrogen fertilizer was applied to winter wheat at growth stage GS40 (boot stage). Three treatments were applied in the experiment: (1) no N was applied, (2) N_{50}, 50 kg ha^{-1}, (3) N_{100}, 100 kg ha^{-1}. The field experiment was established in 2015 and had three seasons: (1) 2015-2016, (2) 2017-2018, (3) 2018-2019.

The plots measured 1.5 m × 5 m area and were randomly arranged in three blocks, with a 0.5 m border between blocks.

Nitrogen and phosphorus fertilizer was applied in autumn ($N_{50}P_{50}$ kg ha^{-1}). Winter wheat was sown (550 seeds per square meter) in October, and harvest in July. Pest and weed control were performed according to local farming practices.

Data collection

Plant height was recorded at full stature as the height to the terminal spikelet less awns. Before harvest plant height was measured in every plot. Grain yield after harvest was reported at 14% moisture content. Grain protein content was determined on the whole grain using Infragrain 9500 analyzer.

Rain-use efficiency (RUE) was calculated according to Gwenzi et al. (2008):

$$\mathrm{RUE} = \frac{\mathrm{GY}}{\text{Total seasonal rainfall}}$$

Nitrogen agronomic efficiency (NAE) was calculated according to Delogu et al. (1998):

$$\mathrm{NAE} = \frac{\text{GY at Nitrogen rate} - \text{GY at 0 Nitrogen rate}}{\text{amount of Nitrogen rate applied}}$$

Nitrogen use efficiency (NUE) was calculated according to Moll et al. (1982):

$$\mathrm{NUE} = \frac{\mathrm{GY}}{\text{amount of Nitrogen rate applied}}$$

Where GY= grain yield.

Statistical analyses

Analysis of variance (ANOVA) and F Test were used to establish the effect of genotype, nitrogen fertilization and season (climatic conditions), and the interactions between these factors. The significance of differences between parameters means were assessed using Duncan's Multiple Range Test at $P \leq 0.05$ level. Direct relationship between rainfall and plant height was analyzed with simple Pearson correlation coefficients.

Climatic conditions

The weather conditions data presented in Table 1 were obtained from a local observation measurement unit located at the Experimental Station. From all three seasons the 2015-2016 was the wettest, followed by the 2018-2019 season, and the 2017-2018 season was the driest. Large amount of precipitations occurred at the end of the winter wheat growing season in 2016 and 2018 in May, June and July. In 2019 it rained heavily in May, after that in June and July the monthly averages were lower than the previous years. From all three seasons 2017-2018 was the warmest, high temperatures at the end of the winter wheat vegetation period determined the speed up of the heading and ripening stages of wheat plants (Table 1).

Table 1. Temperature and rainfall values for the winter wheat vegetation period between 2015-2019, Turda, Romania

Months	Temperature (^{0}C) monthly average				Rainfall (mm) monthly average			
	2015-2016	2017-2018	2018-2019	60 years average	2015-2016	2017-2018	2018-2019	60 years average
October	9.7	11.6	12.7	9.5	45.4	49.2	26.8	35.6
November	6.1	4.9	6	3.9	32	30.8	29.6	28.5
December	0.7	1.0	-0.9	-1.4	6.9	20.7	58.3	27.1
January	-2.8	0.2	-2.2	-3.4	25.0	16.7	46	21.8
February	4.6	-0.3	1.7	-0.9	23.8	33.4	14.7	18.8
March	5.9	3.3	7.3	4.7	47	40.9	12.3	23.6
April	12.4	15.3	11.3	9.9	62.2	26.2	62.6	45.9
May	14.3	18.7	13.6	15	90.4	56.8	152.4	68.7
June	19.8	19.4	21.8	17.9	123.3	98.3	68.8	84.8
July	20.5	20.4	20.4	19.7	124.9	85.7	35.0	77.1
Average	9.12	9.45	9.17	7.49				
Total					580.9	458.7	506.5	431.9

RESULTS AND DISCUSSIONS

Plant height

F test demonstrated that wheat height was significantly influenced by the Genotype by Nitrogen fertilization, by Season and by the interaction between Genotype and Season (Table 2).

For this study the four winter wheat genotypes used had different heights, Arieşan is the taller with a plant height between 90-115 cm, Codru has a plant height between 75-100 cm, Taisa between 90-100 cm and Andrada between 80-95 cm. As Ripberger et al., in 2016, demonstrated that plant height of winter wheat is strongly influenced by the changing of the environmental factors, the plants had higher height under conditions of sufficient moisture. The results of his analysis demonstrated that environmental conditions are responsible for the largest variation of plant height. Voziyan, in 2014, tested 17 winter wheat varieties in the Republic of Moldova for 3 years and concluded that the evaluation of wheat adaptive capacities according to plant height would be more accurate taking into account the dependence of this character on hydrothermal conditions and other biotic and abiotic environmental factors.

Nitrogen fertilization and Season had a strong influence on plant height. The highest plant height was recorded when the nitrogen rate was the highest (Table 3). Also, many authors reported that increasing N level increased plant height (Ali et al., 2000; Iqtidar et al., 2006; Ali et al., 2011).

Environmental conditions had a highly significant effect on plant height, in 2016 when the total rainfall was the highest the plant height increased, in 2018 when the amount of precipitations was the lowest, the plant height recorded was the smallest. The results obtained with the help of Duncan's Multiple Range test demonstrated that the plant height of the four winter wheat cultivars is genetically determinate and this trait is strongly influenced by the environmental factors (Table 2).

As shown in Figure 1 the plant height of all four genotypes from this study was positively correlated with rainfall, the plant height increased as the amount of precipitation was higher. Pearson correlation demonstrated that plant height of Taisa and Andrada was strong correlated with rainfall, and plant height of Arieşan and Codru was moderate correlated with rainfall.

Grain yield

According to F test all the factors of this experience had a significant influence on grain yield, also the interaction between Genotype and Season had a significant influence on yield. Duncan's Multiple Range test demonstrated that Andrada had the highest yield, followed by Codru, Arieşan and Taisa (Table 2).

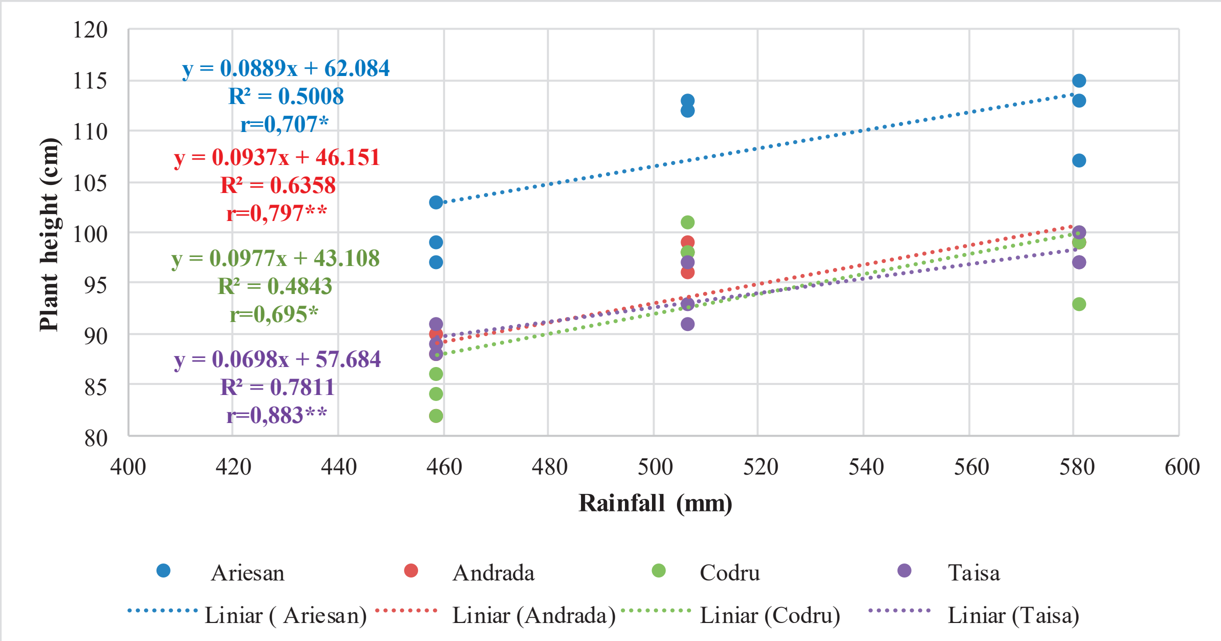

Figure 1. Correlation between rainfall and plant height

Other researchers have reported that generally grain yields increased with increasing nitrogen fertilization rate (Kadar et al., 2019; Guo et al., 2012). Abedi et al. in 2011 demonstrated that different N rates have a significant effect on increasing grain yield. This confirms belonging of winter wheat to the group of plants strongly responding to growth conditions and particularly to fertilization with nitrogen (Buckzek et al., 2018). Our experiment proves the same, the highest grain yield was obtained at the highest rate of nitrogen fertilizer applied (Table 2).

Environmental conditions also play a major role in variation of grain yield, According to Anderson et al,, 2004, even at large rainfall deficits during wheat growth, can be obtained higher yields. As Buckzek et al. (2018) shows, higher yield was obtained when the total rainfall during the growth period of winter wheat was high. Similar to his results the highest yield in our study was obtained in 2016 (total rainfall 580.9 mm), and the smallest in 2019 (total rainfall 458.7 mm) (Table 2).

Grain protein content

The grain protein content is dependent on genotype (Buckzek et al., 2018) but it is also clearly influenced by environmental variables such as nitrogen application, water access and temperature during growth especially through the grain filling period (Daniel and Triboi, 2000; Tea et al., 2004). These factors influence the rate and duration of wheat grain development, protein accumulation and starch deposition (Dupont and Altenbach, 2003). The most effective environmental factor on wheat quality is N fertilization (Abedi et al., 2011).

According to F test Genotype, Nitrogen fertilization and Season had strongly influenced grain protein content of the genotypes used in this study. Also the interaction between Genotype and Nitrogen fertilization, and the interaction between Genotype and Season had a significant influence on this trait (Table 2).

Duncan's Multiple Range test showed that from all four genotypes Arieşan had the highest grain protein content, the other three genotypes had a similar grain protein content. From all three N rates the highest grain protein content was obtained at N 100 kg ha^{-1}, and the environmental condition from 2015-2016 season influenced the most the accumulation of protein in winter wheat grain (Table 2).

Rain use efficiency (RUE) (grain yield/total seasonal rainfall, kg ha^{-1} mm^{-1})

According to Fu et al. (2013), RUE is an accurate indicator of agricultural productivity in relationship to the crop's consumptive use of water, which is defined as the ratio of the net benefits from crop to the amount of water required to produce those benefits. Put simply, it means growing more food or gaining more benefits with less water.

Table 2. Genotype, nitrogen fertilization rate and season effects on winter wheat traits

Factor		Plant height (cm)	Grain yield (kg ha^{-1})	Grain protein content (%)
Genotype (G)	Arieşan	107.74a	6390b	12.98a
	Andrada	94.26b	7010a	11.44b
	Codru	93.48b	6840a	11.47b
	Taisa	93.67b	6040c	11.43b
Nitrogen fertilization (F)	0	95.31c	6140c	10.44c
	50	97.19b	6620b	11.90b
	100	99.36a	6960a	13.16a
Season (S)	2015-2016	101.25a	7310a	12.42a
	2017-2018	89.97b	6990b	11.66b
	2018-2019	100.64a	5420c	11.41c
F Test	G	105.958***	57.828***	62.070***
	F	16.297***	33.572***	406.602***
	S	127.248***	241.474***	35.721***
	GxF	0.322 ns	0,403 ns	5.574**
	GxS	4.256***	35.187***	5.183**
	FxS	0.474 ns	2.730*	1.020 ns
	GxFxS	1.339 ns	0.512 ns	0.722 ns
Mean		97.28	6572	11.83

Means followed by the same letter within a column are not significantly different according to Duncan's Multiple Range test ($p \leq 0.05$).
*,**,*** Significant at the 0.05, 0.01 and 0.0001 probability levels, respectively; ns: nonsignificant.

Duncan's Multiple Range test (Table 3) showed that Andrada and Codru had a significant higher RUE from all four genotype used in this experience, which means that this genotypes produced higher rate of grain yield per unit rainfall than Arieşan and Taisa. The highest RUE was recorded in 2018 when it rained least, the RUE decreased as mean annual precipitation increased as shown by Huxman et al. (2004). Nitrogen fertilization directly influenced RUE, as the rate of nitrogen increased, RUE increased too.

F test proved that all the experimental factors significantly influenced RUE, and the interaction between Genotype and Season had a significant influence on this parameter (Table 3).

According to Mandic et al. (2015), genotypes with improved RUE are particularly beneficial under low rainfall conditions. Our study shows that Taisa had better use of rainfall, followed by Arieşan, Codru and Andrada, since Taisa RUE value was significantly higher (Table 4).

Nitrogen agronomic efficiency (NAE) (kg grain increase kg^{-1} N applied)

This parameter was calculated to assess the potential yield increase in response to different N fertilizer rates, and it is an indicator of the amount of yield per unit of N fertilizer applied, and it is used to evaluate the ability of wheat plant to produce higher yield as related to N fertilization (Tedone et al., 2018).

The ratio of produced grain to N rate of applied fertilizer may be used to estimate NUE, and this ratio has been defined as agronomic efficiency (NAE) (Ladha et al., 2005; Stevens et al., 2005). As this calculation subtracts the yield of the control from the yield of the N treatment plot, this difference method assumes that N fertilization has had no additional positive effects on plant uptake of soil N, and that all other agronomic factors are considered equal between the respective treatments, (Stevens et al., 2005).

The highest NAE was recorded by Taisa which means that the increased yield obtained by Taisa it is due to the N fertilizer rate applied and this cultivar has the highest ability from all four cultivars to increase yield in response to the rate of Nitrogen fertilizer applied (Table 3).

Ayadi et al. (2015) showed that for durum wheat cultivars, the NAE average was more efficient at a lower nitrogen rate N 75 kg ha^{-1}, and less efficient at N 150 kg ha^{-1}. Similar results obtained López Bellido et al. (2001) after studying NAE for bread wheat cultivars at 50 kg ha^{-1} and 150 kg ha^{-1} rates of nitrogen fertilizer applied.

As the research of Mandic et al. (2015), shows that both NAE and NUE decreased when the amount of nitrogen rate increased, our experience proves the same (Table 3). Serret et al. (2008) reported that NAE significantly reduced in the highest N fertilizer level.

F test demonstrated that all the experimental factors and the interactions between these factors had a significant influence on NAE (Table 3).

Nitrogen use efficiency (NUE) (kg grain kg^{-1} N applied)

Nitrogen use efficiency NUE, which represents the kg of grain yield harvested per kg of N fertilizer applied, can be used as an index of total economic outputs relative to the use of all N sources (soil N and applied fertilizer) (Almaliev et al., 2012).

As other studies showed, our research confirms that N fertilization increased NUE, but the highest N level reduced NUE (Hooper, 2010; Almaliev et al., 2012). Somarin et al. (2010) and Noureldin et al. (2013) reported the same, that increased N level reduced NUE (Table 3).

Good et al. (2004) demonstrated that one of the strategies to improve yields is to choose crops with high N use efficiency (NUE) that can produce economic yield under limited water supply. The use of more nutrient efficient crops and varieties is important for maintaining yields while enhancing environmental sustainability. From our experience Andrada and Codru genotypes had the highest value of NUE which means that these cultivars have the ability to use more efficiently the nitrogen to obtain higher yields, they produces higher yields with less nitrogen fertilizer, followed by Arieşan and on the fourth place comes Taisa (Table 3).

Similar to our findings, Almaliev et al. (2012) proved that there are genotypic differences in nitrogen efficiency of wheat cultivars.

F test proved that. NUE was significantly influenced by all the experimental factors, and by the interactions between these factors, except the interaction between Genotype and Nitrogen fertilization (Table 3).

Table 3. Genotype, Nitrogen fertilization level and season effects on rain-use efficiency (RUE), N agronomic efficiency (NAE), and N use efficiency (NUE)

Factor		RUE kg ha^{-1} mm^{-1}	NAE kg grain increase kg^{-1} N applied	NUE kg grain kg^{-1} N applied
Genotype (G)	Arieşan	12.52b	9.24b	98.62b
	Andrada	13.70a	7.57c	107.04a
	Codru	13.36a	7.77c	105.0a
	Taisa	11.81c	11.37a	93.73c
Nitrogen fertilization (F)	0	11.98c	0c	0c
	50	12.96b	9.80a	132.61a
	100	13.60a	8.18b	69.59b
Season (S)	2015-2016	12.60b	4.39c	110.59a
	2017-2018	15.23a	8.69b	107.19b
	2018-2019	10.71c	13.89a	85.52c
F Test	G	43.034***	140.305***	59.626***
	F	33.348***	199.485***	1114.982***
	S	285.360***	1950.230***	180.956***
	GxF	0.476 ns	41.056***	1.544 ns
	GxS	32.367***	194.657***	36.344***
	FxS	2.656*	344.098***	17.165***
	GxFxS	0.551 ns	61.601***	5.285***
Mean		12.84	8.09	90.90

Means followed by the same letter within a column are not significantly different according to Duncan's Multiple Range test ($p \leq 0.05$).
*,**,*** Significant at the 0.05, 0.01 and 0.0001 probability levels, respectively; ns: nonsignificant.

CONCLUSIONS

Nitrogen fertilization and climatic conditions had a strong influence on plant height. The highest plant height was recorded when the nitrogen rate was the highest. Plant height of the four winter wheat cultivars from this study is genetically determinate and this trait is strongly influenced by the environmental factors. Plant height of Taisa and Andrada was strong correlated with rainfall, and plant height of Arieşan and Codru was moderate correlated with rainfall.

Grain yields increased with increasing nitrogen fertilization rate for all four cultivars. Andrada had the highest yield, followed by Codru, Arieşan and Taisa.

The grain protein content is determined by genotype but it is also clearly influenced by environmental variables such as nitrogen application, water access and temperature during growth especially through the grain filling period. From all four cultivars Arieşan had the highest grain protein content.

Andrada and Codru had a significant higher RUE from all four genotype used in this experience, which means that this genotypes produced higher rate of grain yield per unit rainfall than Arieşan and Taisa. The highest NAE was recorded by Taisa which means that the increased yield obtained by Taisa it is due to the N fertilizer rate applied and this cultivar has the highest ability from all four cultivars to increase yield in response to the rate of Nitrogen fertilizer applied. Andrada and Codru had the highest value of NUE, which means that these cultivars have the ability to use more efficiently the nitrogen to obtain higher yields, they produces higher yields with less nitrogen fertilizer, followed by Arieşan and on the fourth place come Taisa. Both NAE and NUE decreased when the amount of nitrogen rate increased.

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DNA-BASED METHODS USED FOR VARIETAL PURITY DETECTION IN WHEAT CULTIVARS

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Abstract

Wheat is a cereal that plays an important role in agriculture, for feed and human food. It is well known that the wheat (Triticum aestivum L.) has a large and complex genome (2n = 6x = 42 chromosomes) that makes difficult the genetic researches. Assessing genetic purity and varietal identification are important topics in wheat seed quality control. Several approaches that can be used to exploit new methods for genetic purity assessment and varietal identification of wheat are currently available in various international laboratories. Techniques based on morphological identification involve intense effort, making it sometimes difficult to verify crops varieties. Using molecular markers in order to determine the purity and variety identification of different seed types seems to be a better approach. Nowadays in the world there are numerous wheat varieties and cultivars with different properties. The molecular markers are not influenced by environment conditions and this makes them play an important role not only for seed purity evaluation but although for research purposes. Taking all that into consideration we can say that DNA-based methods using PCR technique are always useful tools to determine authenticity and purity not only for wheat varieties but for other crops. A better understanding of the factors controlling purity and varietal identification, as well as the effective utilization of new and developing genetics and genomics technologies, have great potential to improve the genetic purity assessment.

***Key words**: genetic purity, molecular markers, varietal identification, wheat.*

WHY PUTTING A HIGH VALUE ON AGRICULTURE TODAY

It is well known that cereals are one of the most important sources of food for human consumption. From the total quantity of cereals produced annually, most of them are intended for food consumption, followed by their use for animal feed, and the rest are processed for industrial use or used as seeds. According to Food and Agriculture Organization (FAO), the 21st century faces multiple challenges in terms of ensuring the need for food, feed and fiber in the conditions of population growth worldwide (FAO, 2010). Thus, there will be an increase of the world population with 34 percent, being expected that by 2050 population will reach approximately 9.7 billion people (FAO, 2009; 2017).

Wheat production ranks third after corn and rice production. In terms of nutritional intake, wheat holds a second place, after rice, given the increased use of corn as animal feed. Nowadays, most of wheat production is used for food consumption, followed by use for animal feed and for industrial use, including biofuels (FAO, 2009; 2018).

In Romania, according to Ministry of Agriculture and Rural Development (MADR, 2018), the area cultivated with wheat registered an increase from 1,975.0 thousand ha in 2007 to 2,109.0 thousand ha in 2018, the average wheat production also recording growth: from 1,541 Kg/ha, as obtained in 2007 and reaching 4,803 Kg/ha in 2018. However, when it comes to wheat production and also other cereals production, can always be badly affected by the influence of climate changes in recent years.

This should be taken into account when it comes to the evolution of cultivated areas and the crops production in Romania.

BASIC REMARKS ABOUT WHEAT GENOME AND COMMON WHEAT EVOLUTION

Common wheat (*Triticum aestivum* L.) known as bread wheat is one of the most cultivated types of wheat. Although the wheat genome has always been considered impossible to sequence due to the large number of repetitive sequences (> 80%) and its size of 17 GB, being five times larger than the human genome (Paux et al., 2008), there are currently numerous studies on wheat genomics.

Triticum aestivum L. has high variability and is a self-pollinating plant species and the most important part about its genome is that common wheat is allohexaploid (2n = 6x = 42 chromosomes, AABBDD) (Novoselskaya, 2015) having six sets of chromosomes, each two sets from three different species (IWGSC, 2014). Thereby, bread wheat contains three subgenomes (ABD) derived from *Triticum urartu* (genome A), *Aegilops speltoides* (genome B) and *Aegilops tauschii* (genome D) (Jizeng et al., 2013; IWGSC, 2014) which sometimes makes its genetic study difficult and last but not least, a challenge to characterize this important genome (Erayman et al., 2004).

Another cultivated wheat species is *Triticum durum* which is tetraploid (2n = 4x = 28 chromosomes) and has two genomes (AABB). Species related to common wheat are rarely cultivated for commercially aim.

Hexaploid and tetraploid wheat species evolved through two important evolutionary processes and according to Baenziger (2016) the progenitor is not known but it seems that it was originally a diploid species. Accordingly, a first evolutionary process consisted of a divergent evolution, when this species evolved into many other diploid species, including *Triticum urartu*, *Triticum tauschii*, barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.). Second evolutionary step consisted of a convergent evolution that was achieved by natural hybridization and spontaneous doubling of the chromosome and made it possible the formation of polyploidy species. One such example is that of *Triticum urartu* (genome A donor) which, following hybridization with an unknown *Triticum* species (genome B donor), and formed *Triticum dicoccoides*, the progenitor of *Triticum durum* (AABB). A second hybridization process that occurred between *Triticum dicoccoides* and *Triticum tauschii* (genome D donor) resulted in the formation of the common wheat, whose genomic constitution is the one mentioned above: AABBDD (Baenziger, 2016).

GENETIC MARKERS CLASSIFICATION AND IMPORTANCE

Genetic markers are allele or DNA sequences that have a known position on the chromosome that contain a specific gene for phenotypic character. They are based on the polymorphism of the gene in terms of phenotypic expression. Genetic markers can be divided into two groups: traditional markers and molecular markers or DNA markers (Raza et al., 2019).

Traditional markers: this group includes three categories of markers: morphological markers, cytological markers and biochemical markers.

Morphological marker are markers that control morphological characters, and can be used to analyze different properties, such as seed shape or flower color, germination mode and other important agronomic parameters.

Advantages of these markers are that their use does not require any biochemical and molecular specific systems, being safe to apply. Amongst disadvantages we can mention that they are in a limited number being affected by different plant germination phases and many other natural elements.

Breeders use successfully these markers in plant breeding program (Raza et al., 2019).

Cytological markers: in terms of cytology we can say that structural features of chromosomes can be obtained by chromosome karyotype and bands, thus cytological markers consist of the combined differences that exist between the structure, size, configuration, sequence and location of the chromosomes, differences that denote the variations in the mode of dispersion of euchromatin and heterochromatin (Jiang, 2013). Cytological analysis can be a useful tool in characterization of wheat species (Kwiatek et al., 2019; Daiyan et al., 2019).

Biochemical markers or allozymes (isozyme) are markers that control biochemical properties. Allozymes analysis has been used for a long time for different purposes such as to mark genetic variability, taxonomy or to study crops genetics and although for plant breeding. DNA mutation involves a replacement of an amino acid and as a consequence modified charges in the electric net of the protein, although the molecule conformation can change.

The advantage of these markers lies in the simplicity of the process, proving themselves to be useful markers for breeders and for genetic studies of plant species.

As a disadvantage we can state the low level of polymorphism and the fact that allozymes are phenotypic markers which implies that they may be affected by external conditions (Kumar et al., 2009).

Molecular markers or DNA markers where extremely used for the determination of genetic diversity in major crops listing wheat (Prasad et al., 2000), rice (Nivedita et al., 2016), maize (Awaludin et al., 2013) and common bean (Debrah et al., 2018).

These markers consist of a small piece of nucleotide sequence and can be analyzed with the help of polymorphisms existing among the nucleotide sequence of various members. Considering that molecular markers are DNA sequence which encodes a particular character or gene of an organism, may be powerful instruments for evaluation of the transmission of hereditary characters, although, speaking of wheat, there is less influence in the transmission of hereditary characters between cultivars, compared to other self-pollinating plants (Raza et al., 2019).

Genetic markers are now frequently used by breeders in breeding programs including here marker assisted selection of cultivars with important agricultural traits, for crops varietal identification or assessment of genetic purity (Zorica, 2010).

MOLECULAR MARKERS AND DNA-BASED TECHNIQUES

The use of molecular markers (DNA-based markers) techniques can offer numerous advantages if we compare with conventional phenotypic approach.

Molecular markers are stable and can be detected in all tissues, they do not depend of plant growth, development and are not influenced by the environment.

Among the criteria to successfully use molecular markers techniques are the following: they have to be polymorphic, equal distributed throughout the plant genome, to highlight genetic differences, to generate reliable markers, be quick, easy to use and not very expensive, the amounts DNA samples needed to be small, to not require prior information about the plant genome or organism, be specific and to have specificity and reproducibility (Agarwal et al., 2008).

Taken into account that no method is perfect, in choosing the applied method it can count the population level at which the studies are performed, as well as the polymorphism provided by the marker used.

Depending on the technique used to identify them, DNA based molecular markers may be divided into two categories: non PCR based and PCR based markers (Agarwal et al., 2008; Kumal et al., 2009).

Non PCR based markers: this first type of markers includes hybridization-based markers such as Restriction Fragment Length Polymorphism (RFLP).

Polymerase chain reaction based markers (PCR based markers): this second category includes among others the Random Amplified Polymorphic DNA markers (RAPD), Amplified Fragment Length Polymorphism markers (AFLP), Microsatellite or Simple Sequence Repeats markers (SSRs), Single Nucleotide Polymorphism markers (SNP), Randomly Amplified Microsatellite Polymorphisms (RAMP) markers, Inter Simple Sequence Repeats (ISSRs), Sequence Related Amplified Polymorphism (SRAP), Sequence Characterized Amplified Region (SCAR), Sequence Tagged Sites (STS), Cleaved Amplified Polymorphic Sequence (CAPS) etc. (Debrah et al., 2018; Raza et al., 2019). In Table 1 are listed some feature comparison of the most used molecular markers.

When talking about PCR-based markers we cannot put aside the development of the PCR technique by Cary Mullis in 1983, which had an important effect on the future development

of molecular biology techniques (Mullis et al., 1986).
In other words we can state that many purposes can be applied for this multitude of DNA markers such as genetic purity evaluation, gene identification, varietal identification, marker assisted selection, quantitative trait loci (QTLs) mapping, genotypic selection, etc. (Bernardo, 2008; Raza et al., 2019; Nadeem et al., 2018).

Table 1. Feature comparison of the most used molecular markers

Characteristics	RFLP	RAPD	AFLP	ISSR	SSR	SNP
Codominant/ Dominant	Codominant	Dominant	Dominant	Dominant	Codominant	Codominant
Reproducibility	High	High	Intermediate	Medium-High	High	High
Polymorphism level	Medium	Very high	High	High	High	High
Makers availability	Low	High	Medium	Medium	Medium	High
DNA quality	High	High	High	Low	Low	High
DNA quantity	High	Medium	Low	Low	Low	Low
Genome abundance	High	Very high	Very high	Medium	Medium	Very high
Analysis costs	High	Low	Moderate	High	High	High
Sequence information	No	No	No	No	Yes	Yes
PCR based	No	Yes	Yes	Yes	Yes	Yes
Visualization	Radioactive	Agarose gel	Agarose gel	Agarose gel	Agarose gel	Automated sequencers

(Kumal et al., 2009; Nadeem et al., 2018).

NON PCR BASED MARKERS TECHNIQUES

Among the first molecular markers technique can be listed methods that used Restriction Fragment Length Polymorphism markers (RFLPs). These markers are based on hybridization. The technique consists in extraction of high quality DNA which is an important step of this method. Another step is the use of restriction enzymes that cut DNA extracted at specific recognition sites resulting in many fragments of different lengths. The fragments are isolated by agarose or polyacrylamide gel electrophoresis (PAGE) (Raza et al., 2019).
Because of their complexity RFLP markers are not quite suitable for breeding (Zhao et al., 2019), they although show low polymorphism which is a major problem for using RFLP markers in wheat, in which case early studies showed that microsatellites markers are more suitable for evaluation of wheat cultivars (Röder et al., 1995). Compared to RFLP markers, SSR markers are more genome specific avoiding confusion created sometimes as a result of the difficulty in results interpretation, because RFLP probes can hybridize more than one positions in wheat genome (Song et al., 2005). Despite all inconvenience related to the use of RFLP markers, it should be mentioned that they were first successfully used in 1975 for genetic mapping of an adeno-virus then for human genome mapping and later on they were choose as markers for plant genomes (Semagn et al., 2006).

PCR BASED MARKERS TECHNIQUES

Randomly Amplified Polymorphic DNA (RAPD) methods involves amplification of genomic DNA by PCR technique, with a single set of primers with an arbitrary nucleotide sequence. The resulting PCR products are isolated by gel electrophoresis. For the success of the technique a condition is that the selected marker should have the content in guanine-cytosine (GC) of at least 40% (Raza et al., 2019).
RAPD markers technique was successfully used for assessment of genetic diversity in wheat (Tamimi & Janabi, 2019) but also for other plants such common bean (Szilagyi et al., 2011), *Amaranthus* species (Popa et al., 2010),

proving that it has strong potential in wheat breeding (Eid, 2019).

Amplified Fragment Length Polymorphism (AFLP) technique merges the Restriction Fragment Length Polymorphism (RFLP) method and PCR technique (Raza et al., 2019). This method turned out to be very sensitive in detecting markers for genetic diversity studies of common wheat cultivars (Arabi et al., 2019; Sadeqi et al., 2019) or among durum wheat cultivars (Roncallo et al., 2019).

Simple Sequence Repeats markers (SSRs) are valuable tools for studying the genetic material of plants.

SSRs markers or microsatellites markers are sequences of one to six nucleotides repeated in tandem. Microsatellites are to be found in the genome but they also exist in other places such mitochondria and we talk about mitochondrial microsatellites and chloroplast when we talk about chloroplast microsatellites (cpSSRs) (Nadeem et al., 2018).

Because of their high polymorphism level they can be easily identified by PCR technique (Raza et al., 2019).

One of their particularities is that being co-dominant markers a comparative analysis of a DNA locus allows us the verification of the similarities between species being able to establishing varietal purity, as well as the phylogenetic relationships between plants (Lakhneko et al., 2016). With their help we could be able to differentiate between the homozygous and heterozygous genotypes (Xin et al., 2005).

SSR markers have been used although to authenticate and identify the genetic purity of various other crops such as maize (Wang et al., 2003), rice (Li et al., 1999; Xin et al., 2005), barley (Owen et al., 2019) etc.

According to various research studies these markers are suitable for uniformity and seed purity assessment of 90% of wheat varieties (Wang et al., 2015), as well as for fingerprinting or varietal identification (Sharma & Singh, 2015; Varshney et al., 2005).

As many studies shows they are used in most areas of crops genetics being as it is stated before high informative, locus specific with co-dominant inheritance.

SSR markers can discriminate between genotypes, being able not only to detect polymorphism but although to estimate genetic diversity in common wheat (Plaschke et al., 1995; Stachel et al., 2000; Prasad et al., 2000) or between durum wheat genotypes (Eujayl et al., 2002; Marzario et al., 2018) being able to differentiate wild species of wheat with A, D and C genomes or to asses genetic variation within these species (Salehi et al., 2018). They are by far the most used markers.

SSRs markers are found to be more informative than other markers in common wheat (Song et al., 2005) and they can be successfully used in another areas such as marker assisted selection (MAS) (Ciucă et al., 2018) with high implications in breeding programs (Stachel et al. 2000; Giura et al., 2019).

Another approach for microsatellite markers is so called sequence-tagged microsatellite site (STMS) when microsatellites markers that contains genomic fragment are cloned and sequenced for primers construction used in PCR amplification. The method involves intense effort, sequence information is needed as long as the characterization and cloning of the probe and mutations cannot be detected outside the target sites (Kumar et al., 2009).

Randomly Amplified Microsatellite Polymorphisms (RAMP) markers techniques, uses SSR primers for genomic DNA amplification that are radiolabeled containing a '5' anchor and '3' repeats. The resulting products are evaluated by agarose gel electrophoresis (Raza et al., 2019).

The technology does not require high costs and RAMP markers have a high level of polymorphism and high genome distribution (Nadeem et al., 2018).

They can be successfully applied and have good potential for studies on various plants molecular characterization (Davila et al., 1999; Guasmi et al., 2012; Salazar et al., 2014).

Single Nucleotide Polymorphism (SNP) markers technique is based on the fact that in many organisms it can occurs variations in the genome sequence in a single nucleotide position (Kumar et al., 2009).

SNPs may be alterations of C/T or G/A or reversions of C/A, A/T, C/G, or T/G depending on nucleotide exchange, in other words addition or deletion of one base (Raza et al., 2019).

The methods using SNPs markers has also gained popularity even though this markers are

only a bi-allelic type (Kumar et al., 2009), mostly because they are present in large numbers in plants and animals. Their frequency in plants may be between one SNPs at every 100-300 base pairs (Nadeem et al., 2018).
Using the SNPs technique it turned out to be useful for varietal identification in crops where polymorphisms is hard to find (Kumar et al., 2009) but although for genomic assisted breeding (Yong et al., 2017) in wheat varieties and cultivars (Vendramin et al., 2019; Körmöczi et al., 2019).

Inter Simple Sequence Repeats (ISSR)
The method uses microsatellites as primers in a PCR reaction with a single primer for many loci in order to amplify usually inter simple sequence repeats of different sizes. The primer for technique can follow the SSR motifs of two up to five nucleotides at microsatellite loci making possible the appearance of several amplification products (Ammiraju et al., 2001).
One of the main advantages of using ISSRs markers is that it does not require the sequence data to obtain the primer and the quantities for template DNA are low, and last but not least they have a random distribution throughout the genome (Kumar et al., 2009).
ISSRs can be used to find markers associated with genes that control important traits thus being important for the selection of varieties with the desired traits (Ammiraju et al., 2001).
This technique was used for varietal identification of different rice genotypes (Dharmaraj et al., 2018), proven to be useful in gene mapping studies (Kumar et al., 2009), and in analysis of wheat genetic diversity (Etminan et al., 2016).
A disadvantage for using ISSRs marker just as for RAPD markers is the reproducibility issues that may occur (Kumar et al., 2009).
Sequence Characterized Amplified Regions (SCARs) markers are DNA fragments that are amplified using PCR technique with specific primers of fifteen to thirty nucleotides sequences cloned from RAPD fragments related to a trait of interest (Kumar et al., 2009).
The SCARs markers are locus specific, easy to use and have highly reproducibility, being used in marker assisted selection and gene mapping studies (Dar et al., 2019).
A disadvantage may be the need of sequence data for the PCR primers construction (Kumar et al., 2009).
Start Codon Targeted (SCoT) markers are markers that are related to short conserved region in plant genes surrounding the translation start codon (ATG).
These markers have been used to evaluate genetic diversity, varietal identification of cultivars, and for quantitative trait loci (QTL) mapping, DNA fingerprinting of different species, including wheat, rice, pea, sugar cane and grapes (Dar et al., 2019; Etminan et al., 2019).
Cleaved Amplified Polymorphic Sequences (CAPS) is a technique that combine RFLP technique with PCR, being able to refer to these markers as PCR- Restriction Fragment Length Polymorphism (PCR-RFLP) markers.
The method consist in amplification of DNA target by PCR using specific primers of twenty-twenty five base pairs then digested with restriction enzymes and products are visualized in agarose or acrylamide gel (Nadeem et al., 2018).
The method requires low quantities of DNA target, has high reproducibility and these markers are codominant but compared to the RFLP technique the polymorphisms is more difficult to find (Kumar et al., 2009).
CAPS marker technique is useful in gene mapping studies or used for marker assisted selection in wheat (Wang et al., 2017; Zhu et al., 2018).

DIFFERENT TRENDS FOR SEED VARIETAL PURITY EVALUATION

Genetic purity testing and varietal identification are important topics in seed quality control. Techniques based on morphological identification involve intense effort, including both material and substantial human resources, making it sometimes difficult to assess the purity and variety of different types of seeds. In this context the use of molecular biology techniques can play an important role both in the identification of varieties much faster and in determining the genetic purity of the seeds in different countries.

Nowadays, the authenticity and purity identification of seeds regularly involve field tests where the purpose is the establishment of Distinctiveness (D), Uniformity (U) and Stability (S) of the variety (DUS test), test that can raise researchable issues (Chakrabarty & Choudhury, 2019). The DUS test takes a long time to perform being sensitive to environmental conditions.

In the 1990s there was a significant development of molecular biology techniques, which made it possible to identify the authenticity and varietal purity in the laboratory, by using molecular markers (Smith & Register, 1998).

The interest of the specialists for such topic is emphasized by many early studies so methods such as detection of polymorphism of prolamin proteins by vertical polyacrylamide gel electrophoresis are used as in order to determine the varietal purity in wheat seeds and triticale since 1997, 1998 (Vyhnánek & Bednár, 2003).

The methods based on PCR technology offer new opportunities for the analysis of varietal purity. The technology uses a small amount of DNA that sometime does not require high purification, and the results can be obtained much faster.

Due to the rapid advancement in biomolecular techniques, the use of molecular markers to test distinctiveness, uniformity and stability comes not only as a necessity but also an addition when their use may be able to replace in near future the morphological observations. This topic is of great interest within the International Union for the Protection of New Varieties of Plants (UPOV) discussions (Debrah et al., 2018).

The use of molecular markers has advantages in terms of method specificity. Their importance in this mater comes from the fact that compared with other biochemical markers such as isoenzymes, they are not dependent on the conditions of the external environment, being independent of the ontogenetic stage of the plant (Vyhnánek & Bednár, 2003) with high implications in plant breeding (Nadeem et al., 2018; Sarika et al., 2017).

Applying these technologies for varietal identification can greatly simplify the seed quality control process, increasing the objectivity and efficiency and at the same time decreasing the material effort in terms of the space necessary for the cultivation as well as the testing time in the field.

Regarding the protection of varieties, seed producers had initiatives on establishment of intellectual property rights in field plants since the end of the 19th century, which led eventually to the adoption of specific legislation regarding the protection of plant varieties in the United States of America but also in some European countries (Correa et al., 2015).

RULES AND REGULATIONS IN FORCE ON VARIETAL PURITY ASSESSMENT

Determining varietal purity by using molecular biology techniques plays an important role also for seed producers in establishing varietal identification of seeds, seeds certification or in case of litigation.

In accordance with the Romanian legislation if there is any suspicion regarding the seeds varietal purity, further analyses can be made. Thereby verification of the seeds varietal purity can be done by methods such as: protein electrophoresis, DNA-polymerase reaction (PCR), Magnetic Resonance Imaging (MRI), ELISA- immunoassay tests and/or other laboratory methods accepted by the institutions and bodies in the European Union and/or internationally (MADR, 2011).

When we talk about seed certification it is well known that the legislative framework is established by normative acts of the Ministry of Agriculture and Rural Development (MADR) in accordance with the requirements of the European Economic Community thereby the varietal purity of the varieties being regulated by law.

According to the law, seeds have sufficient identity and varietal purity or for seeds from a consanguineous line, sufficient identity and purity in terms of their characteristics.

The legislation also mentions the minimum purity of a variety which is examined mainly through field inspections (MADR, 2010).

The minimum purity of a cultivar is different depending on the seed biological category of which belongs as follows: for species such as *Avena nuda*, *Avena sativa*, *Avena strigosa*, *Hordeum vulgare*, *Oryza sativa*, *Triticum aestivum*, *Triticum durum*, *Triticum spelta*, we must have a varietal purity of 99.9% for base category seeds, 99.7% for seeds certified C1, 99.0% C2 certified seed, and for maize lines a minimum varietal purity of 98% (MADR, 2010).

According to the Organisation for Economic Cooperation and Development (OECD) rules, the varietal purity of a hybrid obtained in culture can be verified either in post-control or by additional methods. In order to determine the varietal purity or for varietal identification molecular biology techniques such as DNA or RNA methods can be used. Techniques as RFLP, AFLP, PCR, or the use of molecular markers (SSR or SNP) are very useful when a character is not visible to the naked eye (OECD, 2012).

From all considerations regarding genetic markers listed above one can state the idea that the methods that involve the use of molecular markers are very effective in confirming the purity and authenticity of a plant (Singh et al., 2016).

Currently, one of the techniques used in laboratories to determine the varietal purity, according to The State Institute for Testing and Registration of Varieties (ISTIS) methodology is protein electrophoresis (ISTIS, 2010). The method of determination is in accordance with International Seed Testing Association (ISTA) rules (ISTA, 2019).

In order that the chosen method to be effective in assessing the varietal purity it is important that the tested variety to be accompanied by a reference material. However, for testing purposes, the applicant is not obliged to provide this reference material so, just the varietal purity of the tested sample is being evaluated, which can sometimes be a minus, not taking into account the reference material.

The analysis of the electrophoretic spectra generated by storage proteins allows us to detect varietal impurity for many wheat cultivars (Hassan et al., 2019; Metakovsky et al., 2019; Beom et al., 2018) and may have also implication for plant breeding. Problems may also arise as a result of the fact that the method based on the electrophoresis of storage alcohol-soluble proteins may have its shortcomings so for example in oats, identical spectra makes sometime difficult the use of electrophoresis method for laboratory variety control (Lyubimova & Eremin, 2019).

If we refer to the DUS test to confirm the uniformity we can apply any polymorphic genetic markers, for example, gliadin composition, may be successfully used in hexaploid wheat (Metakovsky et al., 2019).

Among others tests that can be applied for varietal identification we can also list chemical tests. These tests are simple and their application does not require substantial material resources and tests such as standard and modified phenol tests, potassium hydroxide tests and sodium hydroxide tests, growth response of seeds treated with GA_3 could be helpful in assessment of wheat varietal purity (Salem et al., 2019; Raut et al., 2019).

FUTURE ASPECTS, TRENDS AND IMPORTANCE OF USING MOLECULAR BIOLOGY TECHNIQUES

Lately, we have witnessed to a continuous development of molecular biology techniques and starting from RFLP markers to SSRs and SNPs markers we can say that today there are lots of marker associated methods that come in support of researchers, but also of seed producers or breeders. The evaluation of genetic purity of a variety is important both for the registration of the variety and also for its protection. As mentioned above, the current testing system evaluates a number of morphological characters which becomes an impediment in the event of evaluating hundreds of samples. In this context the use of molecular markers becomes of great help.

Among the most used molecular markers we list the SSRs markers that, as mentioned above, have a high degree of polymorphism, high distribution throughout the plant genome and are not affected by external conditions.

A new variety characteristics can be defined by molecular markers patterns and be compared with others patterns from anywhere in the world, to establish its uniqueness (Wang et al., 2015).

The final purpose of the sharp development of the technology we can say that is to create, analyse and not least to manipulate the genetic variation and to develop improved cultivars

(Moose & Mumm, 2008) a marker assisted breeding with high implication in economy (Eathington et al., 2007).
In the last period as a result of the climatic changes and in the conditions of world growth population special attention was paid to the selection of improved cultivars assisted by molecular markers.
For wheat the interest was in obtaining new lines with resistance to various diseases or pests (Simpfendorfer et al., 2013; Goutam et al., 2015), useful in breeding programs to obtain cultivars that are resistant to more than one disease (Miedaner et al., 2019; Ciucă et al. 2015, 2018; Cristina et al., 2015; Miedaner & Korzun, 2012) and/or to counteract the climatic changes the selection of valuable cultivars (Giura et al., 2019; Ciucă et al., 2009; Vlasenko et al., 2019).
As the population increases, it becomes just as important to obtain varieties that have improved content in various substances (i.e. protein content) (Prasad et al., 2003; Cristina et al., 2016).
The marker assisted selection (MAS) has been successfully used for many other crops such rice (Jena & Mackill, 2008; Qing et al., 2019), maize (Prasanna et al., 2010; Awaludin et al., 2013; Raj et al., 2020), barley (Miedaner & Korzun, 2012; Jain et al., 2019; Varshney et al., 2007), sorghum (Afolayan et al., 2019; Kage et al., 2015), the technique was used not only for cereals but also for fruits (Al-Khayri et al., 2018).
As mentioned before the DUS test involves at this point the assessment of morphological characters which are highlighted by field inspections according to the International Union for the Protection of New Varieties of Plants (UPOV) rules. The use of molecular technologies as single way to establish varietal identification in crops is still under discussion as is the use of molecular markers for varietal identification in crops such wheat (UPOV, 2011) or maize (UPOV, 2014).
Since cereals trade is of particular importance for a country's economy, it becomes imperative to protect the breeders rights and the application of molecular markers it becomes important in terms of rapid detection of wheat cultivars and not only (Chun et al., 2018).
Others new and efficient genotyping techniques such as next generation sequencing (NGS) (Adlak et al., 2019), genotyping by sequencing (GBS), chip based NGS, or techniques such as kompetitive allele specific PCR (KASP) (Nadeem et al., 2018; Rasheed et al., 2019) and allele-specific PCR, designate molecular markers as suitable markers for genotyping, making it possible to speed up the process of molecular marker assisted selection techniques in wheat breeding (Raza et al., 2019; Mwadzingeni et al., 2016).
Because the genome complexity in wheat is very high, advances in both bioinformatics and sequencing makes NGS applications in wheat very feasible (Berkman et al., 2012; Bernardo et al., 2020).

CONCLUSIONS

Molecular markers are of particular importance for the development of modern techniques of molecular biology with special implications for breeding program, in germplasm management and improvement of wheat cultivars but also for varietal identification, being able in certain situations to resolve legal dispute.
Thus far, when it comes to wheat and its genome complexity, the need to discover new molecular markers and associated methods is still a goal itself and a challenge for researchers.

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DEALING WITH THE TRANSITION FROM IN LINE ECONOMY TO CIRCULAR ECONOMY - PUBLIC AWARENESS INVESTIGATION IN BUCHAREST

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Abstract

The Circular Economy (CE) concept is an emerging topic. In 2015 European Commission has adopted a circular economy package on waste management. The targets include achieving a recycling rate of 50% by 31 December 2020. The aim of this study is to create a better understanding of public awareness in the promotion of a circular economy in Bucharest, Romania. Under the frame of ERASMUS+ program, in the project entitled "Waste Education Initiative", we developed with EU partners a Circular Economy Guide, for Residents and Students. To assess if the Bucharest citizens support and adopt a "Circular Economy behavior", workshops were carried out amongst Bucharest population, yielding more than 300 voluntarily participants (67% of the total responses were provided by students).

***Key words**: circular economy, public awareness, reducing, recycling, waste generation.*

INTRODUCTION

United Nations forecast suggests that the global population is likely to exceed 11 billion by the end of the 21st century (United Nation, 2016). The economies of the countries have developed a linear model, a "take-make-consume-dispose" pattern of growth (Figure 1).

The linear model is based on the assumption that resources are abundant, available, easy to source and cheap to dispose of (https://eur-lex.europa.eu/resource.html?uri=cellar:aa88c66d-4553-11e4-a0cb-01aa75ed71a1.0022.03/DOC_1&format=PDF).

While growing resources use has increased the welfare of people, resulting in environmental degradation, climate change, and pollution that violate human rights (OHCHR and UNEP, 2012; Velenturf et al., 2018). A growing population impact the demand of production and consumption and that lead to waste overload (Euromonitor International, 2016; Dobbs et al., 2011; Fischer-Kowalski et al., 2011; Morgan et al., 2014; UNEP, 2015; Neligan, 2016; Velenturf, 2018).

In order to meet the new economic, social and environmental requirements, one of the main solutions is to adopt circular economy (Lakatos et al., 2018).

Figure 1. Linear economy scheme - within the "take-make-consume-dispose" approach (Thibaut Wautelet, 2018)

According to the definition used by the Ellen Mac Arthur Foundation, a circular economy is an economy that is restorative and regenerative by design and aims to keep products, components and materials at their highest utility and value at all times (https://www.ellenmacarthurfoundation.org/circular-economy/what-is-the-circular-economy).

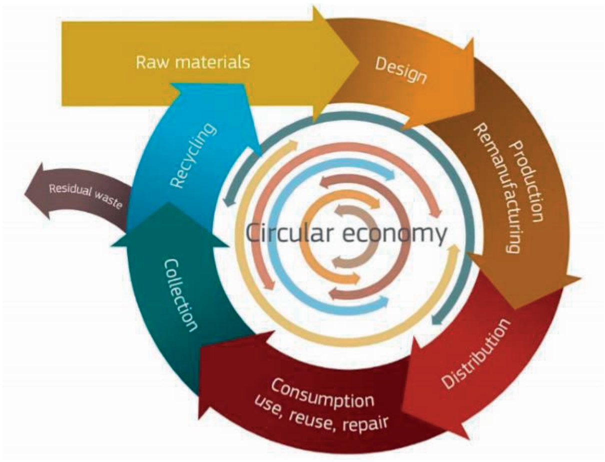

Figure 2. Conceptual diagram of Circular Economy (COM 398 final/2, 2014)

Ellen McArthur Foundation sees the circular economy inspired by nature, where everything is used and nothing is wasted.

In December 2015 European Commission published an action plan for the circular economy implementation, *Closing the loop EU action plan*. This plan aims to support the EU transition from linear to circular economy (https://www.eea.europa.eu/policy-documents/com-2015-0614-final).

The Circular Economy is a system built on *reduction, reuse and recycling* approach. In the circular economy model, material cycles are closed, because waste is considered as valuable resource. Used and broken products can be repaired and reused many times, though some products can be easily reused directly and only residual streams are recycled.

For a circular economy correct implementation it is essential to recycle all materials from waste streams in order to close the loop during the whole life cycle of a product (Figure 2).

The waste management is one of sector indicated by the Commission in the communication of 2015. An effective waste management programme would result in an improved quality of life, reducing the impact on human health and the environment (Olaru, 2016).

The quality of a country's waste management improves by moving up the waste hierarchy (Figure 3). As a first priority, waste should be avoided. Waste disposal should be phased out and, where it is unavoidable, it must be adequately controlled to be safe for human health and the environment (https://ec.europa.eu/jrc/en/research-topic/waste-and-recycling).

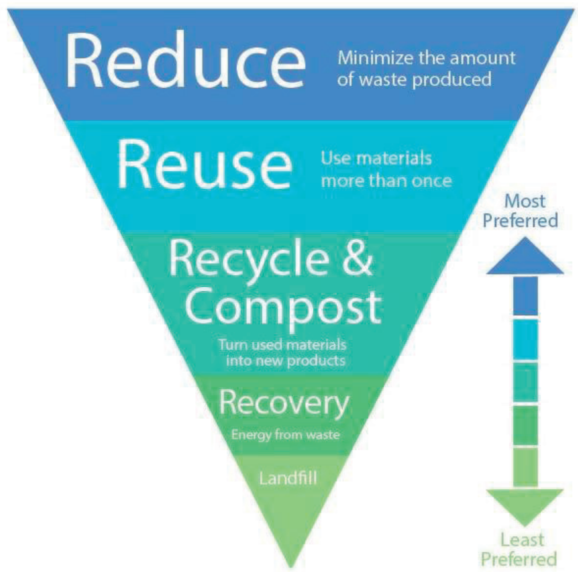

Figure 3. Waste hierarchy as stated by Directive 2008/98/EC on waste

According to Directive 2008/98/EC waste is defined as "any substance or object which the holder discards or intends or is required to discard".

European Union has a main objective: by 31 of December 2020, EU countries will have a level of preparedness for re-use and recycling of at least 50% of the total amount of waste generated (Cecere and Corrocher, 2016).

Figure 4 show the amount of municipal solid waste produced by the EU member states in 2017. Romania generated the smallest amount of waste per capita, 272 kg. The largest amount of waste per capita was generated by Denmark, 781 kg (Eurostat, 2017).

However, according to Eurostat, Romania recorded a very low recycling rate of municipal waste of only 14% in 2017. Romania's recycling rate is one of the lowest in the EU with most of the waste going to landfill so, there is a risk of missing the EU 2020 recycling targets. Unfortunately, Romania is even a negative example in terms of waste management (Feodorov, 2018).

In Figure 5 is relevant highlighted the low quantity of municipal solid waste that Romania recycles, compared to EU average. Other states manage to recycle around one third of municipal waste - Poland 34%, Bulgaria and Hungary - 35%. Germany is the European Union champion for recycling with a 68% recycling rate.

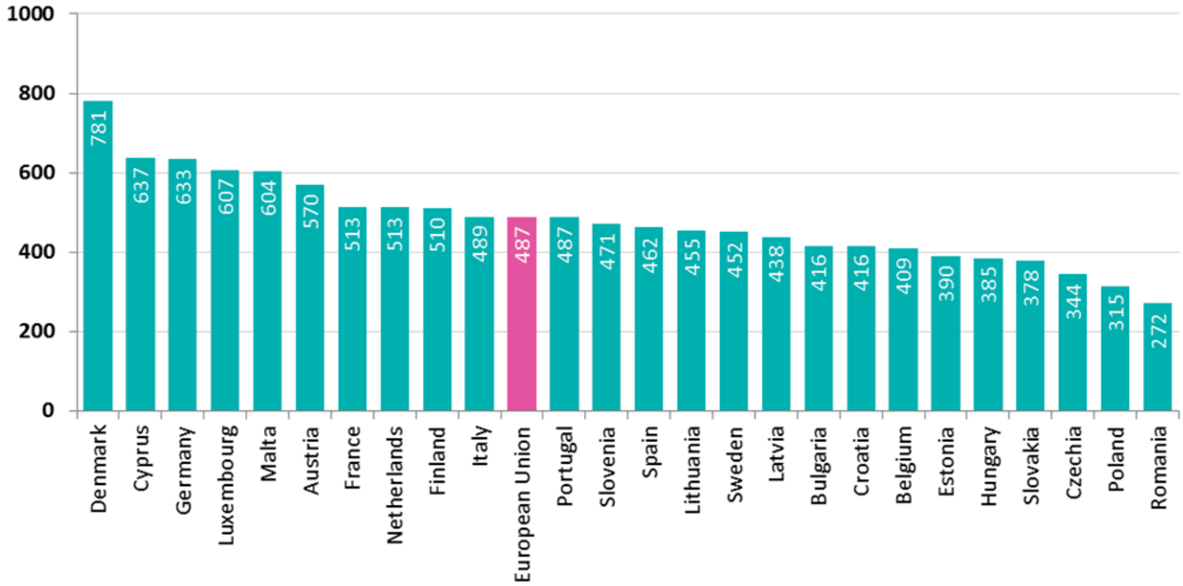

Figure 4. The amount of municipal solid waste generated in EU Member States in year 2017, kg per person (Eurostat)

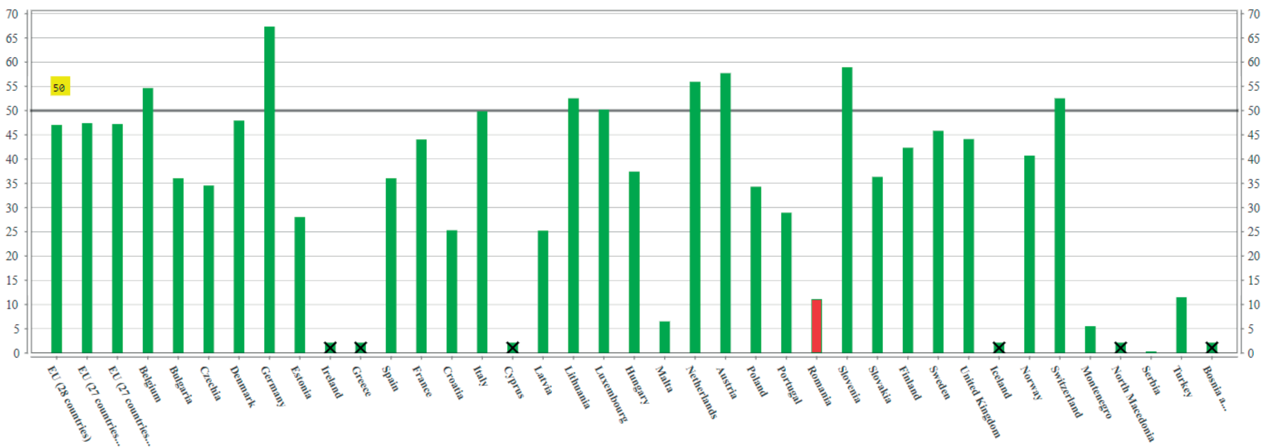

Figure 5. Country comparison - Municipal waste recycled in Europe, % (Eurostat)

Circular economy systems keep the added value in products for as long as possible and eliminate waste generation (http://ec.europa.eu/environment/circulareconomy/index_en.htm). Turning waste into a resource is an essential part of increasing resource efficiency and closing the loop in a circular economy vision.

MATERIALS AND METHODS

In line with these notions and since Circular Economy is a relatively new concept in Romania, a research plan was designed and carried out amongst Bucharest population, to assess their views and support of CE practices. Under the frame of ERASMUS+ program, in the project entitled "Waste Education Initiative", we developed and propose together with the EU project partners a *Circular Economy Guide for Residents and Students* (https://www2.mmu.ac.uk/environmental-science-research/waste-to-resource-innovation-network/activity/erasmusplus-waste-education-initiative/waste-education-teaching-guide-and-resources/) The guide provides general information about the principles of the Circular Economy (Figure 6) and why is the Circular Economy so important. In the end, the guide provides examples of Circular Economy in Bucharest, Romania (https://www2.mmu.ac.uk/media/mmuacuk/content/documents/w2rin/5756-R4GM-IO4-Romanian-v3.pdf).

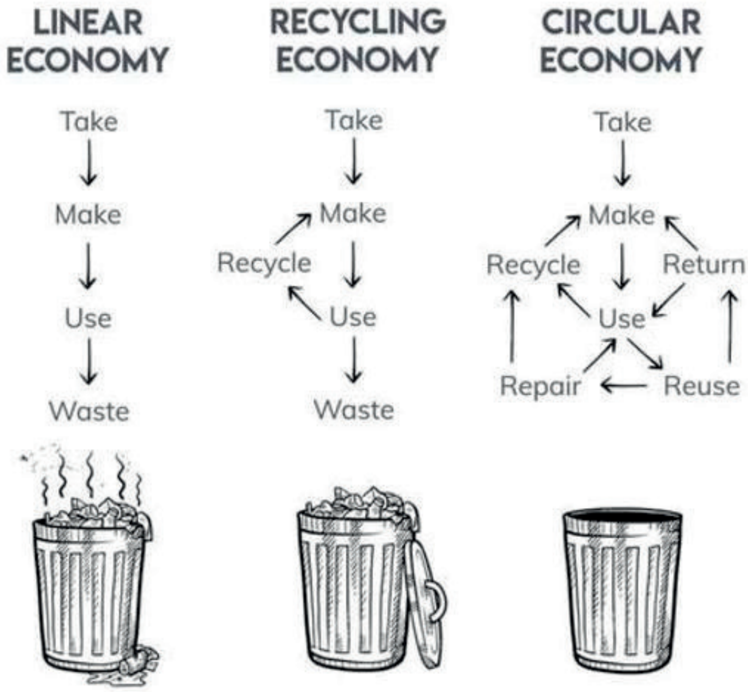

Figure 6. Circular economy versus linear economy (https://thercollective.com/blogs/r-stories/circular-economy-vs-linear-economy)

In November and December 2019, we had organized six workshops participating 122 voluntarily residents from Bucharest.

The workshops were organized in different locations: in residential communities, in cafes destined for such meetings and in the University of Agronomic Sciences and Veterinary Medicine of Bucharest (Figures 7 and 8).

The necessity of a circular economy has been highlighted by *The Circular Economy Guide, for Residents and Students* in a flyer form. We also create a second flyer *Selective waste collection in Romania* which provide information on how to properly do the selective/separate collection of municipal waste on 4 fractions: plastic/metal, paper/cardboard, glass and mixed waste (Figure 9).

Figure 7. Residents workshops in a coffee house

Figure 8. Residents workshops in USAMVB sport gym

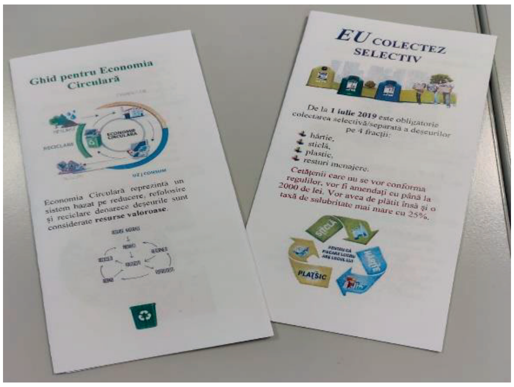

Figure 9. Circular Economy Guide, for Residents and Students and Selective waste collection in Romania (flyer form)

Currently the selective collection of municipal waste system is implemented very slowly due to the difficulties in setting up logistics and infrastructure, as well as for the lack of continuous educational activities.
In this context for student's acknowledgement we organized three workshops in University of Agronomic Sciences and Veterinary Medicine of Bucharest with a total of 245 voluntarily participants (Figures 10 and 11).

Figure 10. Students workshops

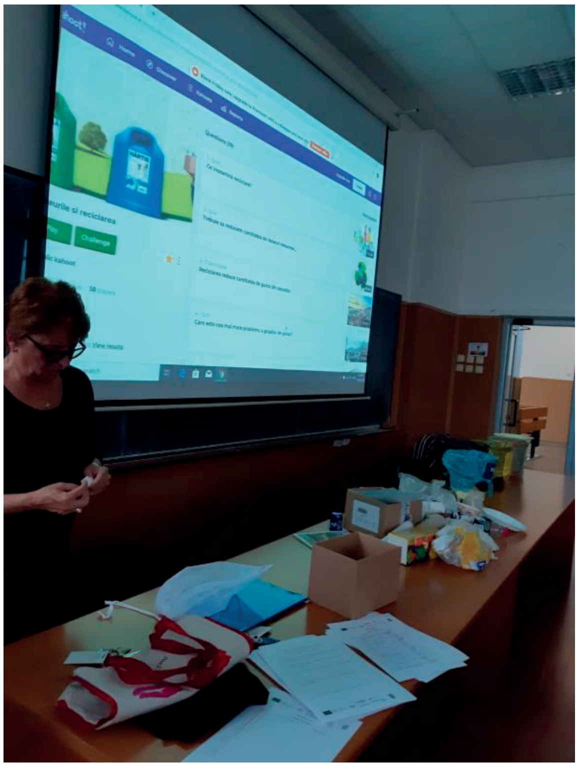

Figure 11. Students workshops

Finally, the research data from residents and students were collected through a feedback form which included 4 questions:

1. Has this guide increased your knowledge about the Circular Economy?
2. Do you think this guide will have an impact on how do you recycle?
3. How do you think the CE guide could be improved?
4. Any other comments?

RESULTS AND DISCUSSIONS

In total, a number of 367 citizens from Bucharest attended to the workshops organized to promote waste education and CE (Figure 12).

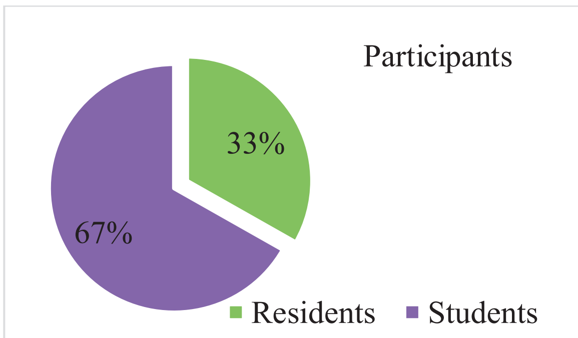

Figure 12. Workshops participants

After each meeting participants completed the workshop feedback form answering to the fourth questions presented above.

Regarding the first question: *Has this guide increased your knowledge about the Circular Economy?* The results indicate that the participants have limited awareness and a poor understanding about the CE and the guide increased their knowledge about the Circular Economy (Figure 13).

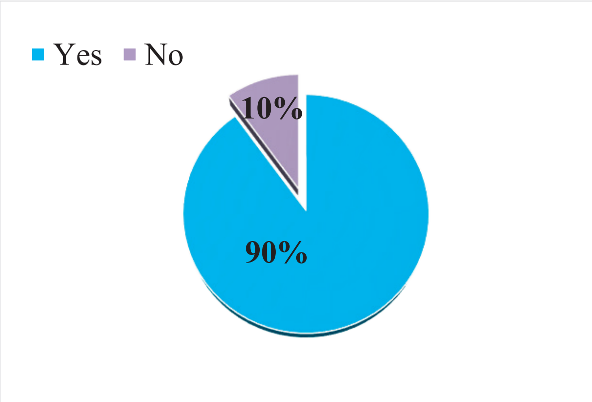

Figure 13. Participants answers diagram to the first question

To the second question: *Do you think this guide will have an impact on how you recycle?*, the answers were 100% completed with YES and it shows how this guide helped the residents and students to understand the impact of their attitude about consumption, resources and waste generation and why is important recycling for the circular economy growth.

To the third question: *How do you think the CE guide could be improved?* there was a general consensus that the information provided are sufficient.

To the fourth question: *Any other comments?*, approximately 59% of respondents testify, however, that they are confused when it comes to selective waste collection, which is not surprising given the fact that some packages are made from several materials that should be separated. About 62% of all respondents consider that the main barriers on circular economy development are: weakness of public awareness, lack of financial support, the gap between policy-making and practical activities. The resident and students request a sustainable infrastructure for correct collection of waste. All people interviewed observed a necessity for waste management education. Especially students consider useful more practical information about CE and waste management designed and presented in a way which will include individuals and society's consumption features apart from being only aware of teaching instructions to respect the natural environment. In this sense, changing individuals and society's consumption habits is the most important additional target to be made for nature and a safe environment for the next generation.

CONCLUSIONS

Compared with the EU member states, even if Romania generated the lowest amount of municipal solid waste in 2017 (272 kg per capita), the 14% of recycling rate shows the bizarre situation Romania is facing because of the inconstancies between waste policies, education, infrastructure available and whole waste management system. Given that the target to be reached in December 2020 is 50%, it is indubitable now that implementation of the circular economy enables reduction of generated waste and increase of waste reuse indices, and therefore might actually improve natural environment conditions. Residents and students answers reflect the Romanian waste management system need in terms of information/education, infrastructure and facilities availability.

The study demonstrates that a *Circular Economy Guide, for Residents and Students* is very useful and provide appropriate information for people to understand the urgent need of the linear economy transition towards CE, from the classic production and consumption model, which is still widely used in Romania to a circular material loop.

Consistently, circular economy presents a practical solution to the earth's resource limitations and to succeed, it is imperative for consumers to engage in circular economy practices by innovating a very different and complex mechanism of materials and energy circuit for required products.

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EVALUATION OF PRODUCTIVITY AND DROUGHT TOLERANCE OF OIL-SEED FLAX (*Linum usitatissimum* L.) VARIETIES DEPENDING ON THE CONDITIONS OF HUMIDIFICATION AND MINERAL NUTRITION IN THE SOUTH OF UKRAINE

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Abstract

The goal of the study was to determine drought tolerance of varieties of oil-seed flax and to improve its cultivation technology through the optimization of mineral nutrition in dependence on soil humidification with connection to the changes of climate in the South of Ukraine. To achieve the goal we performed three-year field experiment with the crop in regard to the design of the study, and investigated such factors as variety (Evryka, Orfei, Vira), mineral nutrition background (N_0P_0, $N_{45}P_{60}$, $N_{60}P_{60}$, $N_{90}P_{60}$), soil humidification conditions (irrigation, rain-fed). The maximum yields were provided by the variety Evryka (2.36 t ha^{-1}) in the irrigated conditions, and the variety Vira (1.47 t ha^{-1}) in the non-irrigated conditions at the application of $N_{90}P_{60}$. The best yield of oil was provided by the variety Vira (0.97 t ha^{-1} and 0.57 t ha^{-1} with the application of $N_{60}P_{60}$ in the irrigated and non-irrigated conditions, respectively). The main indices of drought tolerance, namely, mean productivity (MP) - 1.30, yield stability index (YSI) - 0.55, yielding index (YI) - 106, stress tolerance index (STI) - 0.47, revealed that the variety Vira was the best one among the studied varieties of oil-seed flax. Multiple linear regression analysis revealed general tendency to the increase in oil-seed yield by 6.431 kg with the increase of irrigation rate by every 1 mm; by 4.548 kg per every 1 kg ha^{-1} of Nitrogen fertilizers; by 1.228 kg per every 1 kg ha^{-1} of Phosphorus fertilizers, respectively.

Key words: *change of climate, cultivation conditions, seed yield, weather conditions, yield of oil.*

INTRODUCTION

In recent years due to the diversification of grain production there is an increase in the sowing areas under the oil crops among which oil-seed flax has a special place. Oil-seed flax (*Linum usitatissimum* L.) is used as a raw material for production of fast-drying oil for chemical industry (Nykter et al., 2006), linoleum, as a food and fodder additive (Bernacchia et al., 2014; Kajla et al., 2015), directly for nutrition and in the form of flour in bakery.

It was determined that flax was known as a fiber and oil plant in the ancient East, and it was introduced in agricultural practice earlier than cotton-plant (Herbig & Maier, 2011; Valamoti, 2011). The earliest findings of the seeds of flax crop are dated 4900-8700 BC (Vaisey-Genser & Morris, 2003; Zohary et al., 2012; Harris, 2014). Other reports certify that oil fiber with the age of nearly 30 thousand years was discovered (Kvavadze et al., 2009). Geography of the findings of flax remnants is very wide. Oil-seed flax could be cultivated in different regions of Ukraine, and it does not concede by the profitability level to other oil crops.

The advantage of oil-seed flax cultivation is, firstly, its drought tolerance that gives an opportunity to obtain yields of 1.2-2.5 t ha^{-1} every year. Secondly, it has a short vegetative period (80-105 days) that allows harvesting of flax in the end of July, and as a result, it is one of the best previous crops for winter cereal crops. Thirdly, tolerance to unfavorable weather and climatic conditions, especially seedlings are tolerant to the spring frosts, and the crop is seed-cast and lodging resistant. Besides, oil-seed flax has easy cultivation technology, does not require application of insecticides and fungicides owing to the absence of specific insects and diseases in the Steppe zone of Ukraine, it is not exigent to soil fertility and could be cultivated without application of fertilizers as an ensuring crop for the occurrence of winter cereals re-sowing.

Drought tolerance is a capacity of plants to endure significant dewatering and overheating maintaining normal growth, development and reproductive capacity. The problem of drought tolerance is actual for many regions of our country and the world because of changes in the parameters of climate to its warming that is accompanied by drought. Scientists believe that oil-seed flax is a good drought-resistant crop (Chekhov et al., 2007; Sheremet et al., 2014). However, some scientists state that notwithstanding biologically caused high drought-tolerance and plasticity, oil-seed flax in the South of Ukraine suffers from the lack of moisture (because of high transpiration coefficient of the crop - 420-690) and is very responsible to irrigation (Gobelyal, 2007; Rudik, 2013). The root system of flax is comparatively poor developed and shallow, but its suction force is very high. Most moisture is used from the soil layer of 0-60 cm (Johnston et al., 2002). The feature of the root system of flax is its unstoppable growth in depth to the end of vegetation. This provides the plants with an opportunity to assimilate moisture after flowering from the deeper soil layers and provides better standing of drought in comparison to other crops. Besides, flax is supposed to be the best option for no-till cropping systems (Lafond et al., 1992).

The maximum yield of seeds is provided by flax when the crop has enough precipitation or irrigation amounts (about 50 mm) during the period from budding to the end of flowering at the moderate temperatures (Zinchenko et al., 2001). The researches proved that in average at the expense of irrigation seed yield of oil-seed flax increases by 34.9%. Flax is very responsive to the application of fertilizers. Strengthening of mineral nutrition ($N_{90}P_{60}K_{60}$) is accompanied by the increase in the yield by 41.0-43.3% (Rudik, 2016).

So, the thoughts of scientists differ, some of them state that flax is a drought-tolerant crop while others convince that oil-seed flax suffers from the lack of humidification in the South of Ukraine.

The aim of our study was to determine the drought tolerance of oil-seed flax varieties and to improve their cultivation technology through the optimization of mineral nutrition in dependence on the soil humidification conditions in Southern region of Ukraine in connection with the global changes of climate.

MATERIALS AND METHODS

The study was carried out at the experimental field of Askania State Agricultural Research Station of the Institute of Irrigated Agriculture of NAAS in 2015-2018. The study was conducted in three replications by using the systematic design. The cultivation technology of oil-seed flax was common for the conditions of the South of Ukraine, excluding the studied factors. The previous crop was winter wheat. Soil tillage was performed by plowing on the depth of 20-22 cm. Application of mineral fertilizers was performed with accordance to the design of the study. The sowing rate of all the studied varieties of the crop was 5 million seeds ha^{-1}. Sowing was conducted by the seed drill “KLEN-1.6”. Four waterings with the rate of 30 mm were carried out during the vegetative period of the crop by the means of an irrigation machine “Zimmatik”. Plant care included the application of herbicide Bazahran (active substance *bentazon*, 480 g L^{-1}) in the dose of 1.0 L ha^{-1} at the height of the crop 3-10 cm, and the application of insecticide Razit (active substances include *imidacloprid*, 140 g L^{-1}, *acetamiprid*, 160 g L^{-1}, *cypermethrin*, 100 g L^{-1}) in the dose of 0.2 L ha^{-1} at the stage of the crop’s young growth. The yield was harvested by using a self-propelled combine “Sampo-130”. The oil content in the seeds was determined by the method of extraction in the apparatus of Soxhlet with accordance to the State Standard DSTU 7577:2014 (Ministry of Agrarian Policy and Food of Ukraine, 2016). Statistical data processing was performed by the means of AgroStat add-in for Microsoft Excel (Ushkarenko et al., 2014). The difference between the variants is significant at the probability level of 95%, and it is proved by the results of multi-factor analysis of variance (ANOVA) test with calculation of the least significant difference (LSD_{05}). Correlation analysis was performed within Microsoft Excel software by the standard methodology of calculations (Ushkarenko et al., 2014). Regression model of the yield depending on irrigation and mineral fertilization was carried out using the multiple regression analysis procedure within Microsoft Excel software.

The soil of the experimental field is dark-chestnut clay-loamy slightly-solonized with the humus content of 2.15-2.30% in the arable layer. The bulk density of the soil layer 0-40 cm is 1.2-1.3 g cm^{-3}, the wilting point in the layer 0-40 cm is 7.8-9.8%, and the water-holding capacity in the soil layer of 0-70 cm is 20.5-22.4%. Groundwater lays deeper than 15 m. The water for irrigation was taken from the Kakhovka irrigation system, which is characterized as suitable for irrigation without limitation.

The study included an investigation of the factors: A - humidification regime (with or without irrigation); B - varieties of oil-seed flax (Evryka, Orfei, Vira); C - mineral nutrition background (N_0P_0, $N_{45}P_{60}$, $N_{60}P_{60}$, $N_{90}P_{60}$).

Evryka is a variety of linseed flax recommended for cultivation in the zones of Forest-Steppe, Steppe, Polissya. The plants are characterized with high tolerance to lodging, seed falling, capsules cracking, moderate tolerance to diseases. The height of the plants is 57-62 cm, the stem is round with the diameter of 3-4 mm. The duration of period of vegetation is 81 days. 1000 seed weight is 7-8 g. The variety is included in the State Register of Plants and Varieties of Ukraine since 2004.

Orfei is a variety of oil-seed flax with high drought and lodging tolerance. Potential productivity of the plants is high. The height of the plants is 52-55 cm. The duration of period of vegetation is 84-86 days. 1000 seed weight is 7-9 g, the oil content in the seeds is about 44.8%. The variety is included in the State Register of Plants and Varieties of Ukraine since 2002.

Vira is an oil-seed flax variety with high tolerance to lodging and seed falling. The duration of period of vegetation is 83 days. The yield of oil is nearly 1.10 t ha^{-1}. The variety is included in the State Register of Plants and Varieties of Ukraine since 2009.

The methods used in the study - field method for the determination of the dependence of the studied object from anthropogenic factors and seed productivity, analytical method for the estimation of the crop cultivation conditions, statistical method for the calculations and evaluation of the significance of the obtained results.

Drought tolerance was determined by the index of stress tolerance (TOL) as the difference between the yield in stress (Ys) and non-stress (Yp) conditions, and between the mean productivity (MP) of Ys and Yp (Rosielle & Hamblin, 1981) by using the following formulas:

$TOL = Y - Ys$

$MP = (Yp+Ys)/2$

where: *Yp* is the yield in the optimum conditions; *Ys* is the yield in the drought stress conditions.

We also calculated the index of drought stress susceptibility (DSI), which characterizes the level of susceptibility of the genotype to different stress factors, especially – drought (Fisher & Maurer, 1978), and the index of stress tolerance (STI) by using the formulas:

$DSI = (1 - Y/Yp)/D$

$D = 1 - X/Xp$

$STI = (Yp \times Ys)/Ypm^2$

where: *Y* is the yield in the drought conditions; *Yp* is the yield in the optimum conditions; *D* is the intensity of drought; *X* and *Xp* is the level of yield of all the varieties under the influence of drought and without it, respectively; *D* value usually fluctuates within 0 - 1.

We also used geometrically mean productivity of the yield of the studied varieties to evaluate their drought tolerance (GMP) (Yücel & Mart, 2014) by the following equation:

$GMP = \sqrt{Yp \times Ys}$

where: *Yp* is the yield in the optimum conditions; *Ys* is the yield in the drought stress conditions.

The index of yield stability (YSI) and the index of yield in the stress conditions were calculated by the ratio of the stress-influenced yield to the mean yield of the studied variety (Bouslama & Schapaugh, 1984; Gavuzzi et al., 1997):

$YSI = Ys/Yp$

where: *Yp* is the yield in the optimum conditions; *Ys* is the yield in the drought stress conditions.

RESULTS AND DISCUSSIONS

General feature of the climate of southern Steppe zone is insufficient amount of precipitation, low relative air humidity, frequent dry-winds, warm autumn and winter, and prolonged period without frosts. The climate of the South of Ukraine is continental, hot, dry. We used the data of observations of Kherson agrometeorological station, which have been carried out since 1882, to evaluate the weather conditions of the years of the study (Table 1).

Meteorological conditions of 2016 were the most favorable for the growth and development of oil-seed flax plants both in the irrigated and non-irrigated conditions. During the period of March, April and May 130.1 mm of precipitation was fixed. The maximum amount of rainfall was observed in the III decade of April and I decade of May - 78.4 mm. There was 65.9 mm of precipitation in June. This amount surpassed the long-term mean norm 1.5 times (65.9 mm in comparison to 46.0 mm) but it was still insufficient for the formation of high yield of the crop in the rain-fed conditions. July was characterized by very low amount of rainfall (only 20 mm) that had no considerable effect on the oil-seed flax crop in the end of its vegetation.

Table 1. Weather conditions during the period of vegetation of the studied oil-seed flax varieties, average for 2016-2018

Month	Index	Years of the study			Average air temperature, °C	Average sum of precipitation, mm	Long-term mean indices
		2016	2017	2018			
March	Average daily temperature, °C	6.1	4.5	1.5	4.0	-	2.2
	Precipitation, mm	25.3	10.2	35.1	-	23.5	26.0
April	Average daily temperature, °C	12.4	7.6	12.9	10.9	-	9.6
	Precipitation, mm	41.7	81.8	2.7	-	42.1	28.0
May	Average daily temperature, °C	15.9	13.9	19.5	16.4	-	15.6
	Precipitation, mm	63.1	25.8	13.0	-	33.9	38.0
June	Average daily temperature, °C	21.5	19.7	22.4	21.2	-	20.0
	Precipitation, mm	65.9	8.0	23.0	-	32.3	46.0
July	Average daily temperature, °C	23.9	21.9	24.1	23.3	-	22.4
	Precipitation, mm	20.0	80.0	61.5	-	53.8	42.0
August	Average daily temperature, °C	24.6	22.9	25.0	24.2	-	21.6
	Precipitation, mm	88.5	33.0	15.0	-	45.5	35.0
September	Average daily temperature, °C	17.4	18.6	18.7	18.2	-	16.4
	Precipitation, mm	28.2	32.0	14.0	-	24.7	28.0
October	Average daily temperature, °C	8.5	7.1	10.5	8.7	-	9.6
	Precipitation, mm	46.3	49.3	21.0	-	38.7	26.0

Table 2. Field seedling rate and plant height of the oil-seed flax varieties depending on the application of mineral fertilizers at the irrigated and rain-fed conditions, average for 2016-2018

Humidification conditions	Variety	Mineral nutrition background	Plant height, cm	Field seedling rate, %	Plant density, million ha^{-1}
Irrigation	Evryka	Control (N_0 P_0)	55.8	90	4.5
		N_{45} P_{60}	57.4	90	4.5
		N_{60} P_{60}	60.3	90	4.5
		N_{90} P_{60}	61.3	91	4.5
	Orfei	Control (N_0 P_0)	59.9	92	4.6
		N_{45} P_{60}	62.3	91	4.5
		N_{60} P_{60}	63.1	93	4.6
		N_{90} P_{60}	64.8	92	4.6
	Vira	Control (N_0 P_0)	57.2	95	4.7
		N_{45} P_{60}	58.6	95	4.8
		N_{60} P_{60}	59.7	95	4.7
		N_{90} P_{60}	63.2	95	4.8
Rain-fed	Evryka	Control (N_0 P_0)	46.5	96	4.3
		N_{45} P_{60}	47.5	86	4.3
		N_{60} P_{60}	48.8	86	4.4
		N_{90} P_{60}	51.3	87	4.3
	Orfei	Control (N_0 P_0)	47.5	87	4.4
		N_{45} P_{60}	49.0	89	4.5
		N_{60} P_{60}	50.1	90	4.5
		N_{90} P_{60}	50.7	89	4.5
	Vira	Control (N_0 P_0)	46.5	90	4.6
		N_{45} P_{60}	48.8	92	4.6
		N_{60} P_{60}	49.8	92	4.6
		N_{90} P_{60}	50.6	92	4.6
LSD_{05}		Factor A	0.34	1.65	0.12
		Factor B	0.28	0.96	0.13
		Factor C	0.40	0.97	0.08

Beginning of the spring of 2017 was in usual terms. During March, April and May 117.8 mm of rainfall came to the experimental field, 81.8 mm of them - in April. April was humid but comparatively cold: temperature regime of April and May was lower than the long-term mean norm by 1.7-2.0ºC, and on 19th of April there was snow-cover of 15-20 cm and temperature decreased to 0ºC that negatively affected the plants of oil-seed flax and caused the stagnation of their growth. The 3rd decade of May was characterized by the by the gradual increase of mean daily air temperature.

The spring of 2018 was late. Snow-cover was on the ground until the end of March. Such weather provided good storage of productive moisture in the soil (up to 200 mm). Mean daily temperatures from April began to increase, and at the beginning of May reached the values of 25-35ºC. May was very hot with low rainfall amount (13 mm against 38 mm as the long-term mean norm). The absence of moisture accompanied by the enormously high temperatures in 2018 had a negative effect on the yield of the crop in the non-irrigated conditions (Table 4). The yields on the fertilized plots in the irrigated conditions fluctuated within 2.18-2.48 t ha^{-1}, and in the rain-fed conditions it was just about 0.79-1.00 t ha^{-1}, or 1,39-1,48 t ha^{-1} lower.

The analysis of weather conditions showed that 2016 was the most favorable year for the growth and development of oil-seed flax: 379 mm of productive moisture came into the soil during the vegetative period of the crop. The level of moisture income was slightly lower in 2017 - 320 mm, and considerably lower in 2018 - just 185 mm. We should mention that 2018 was very dry year even for the conditions of the arid Steppe zone.

The results of the three-year study testified that the plants of oil-seed flax differed by their height within the variety in dependence on the dose of fertilizers applied and conditions of soil humidification.

The difference on the fertilized variants in the irrigated conditions between the lowest and the highest plant was: at the variety Evryka - 3.9 cm, Orfei - 2.5 cm, Vira - 4.6 cm, respectively. With the increase of Nitrogen fertilizer dose the height of the plants increased. The maximum plant height was observed on the variants with the application of $N_{90}P_{60}$ (61.3-63.2 cm). The above-mentioned difference in the height of the plants was diminished in the variants with no irrigation, and it was 3.8, 1.7, 1.8 cm for the varieties Evryka, Orfei and Vira, respectively. However, the tendency regarding the fertilization saved: the highest plants were on the plots with $N_{90}P_{60}$ - 50.6-51.3 cm (Table 2).

Humidification conditions significantly affected the formation of the plant height of oil-seed flax. The plants were in average 10 cm higher in the irrigated conditions than in the rain-fed ones.

The best indices of field seedling and plant density were observed at the plants of the variety Vira with the indices of 95% and 4.8 million of plants ha^{-1} in the irrigated conditions, and 92% and 4.6 million of plants ha^{-1} in the rain-fed conditions, respectively. The variety Orfei had worse indices of seedling rate and plant density (93% and 4.6 million of plants ha^{-1} in the irrigated conditions, and 89% and 4.5 million of plants ha^{-1} in the non-irrigated conditions, respectively), and the variety Evryka had the worst indices of 91% and 4.5 million of plants ha^{-1} in the irrigated conditions, and 87% and 4.3 million of plants ha^{-1} in the rain-fed conditions, respectively. Almost the same tendency of the seed seedling was observed in the conditions of laboratory.

The conditions of cultivation affected significantly on the formation of the elements of the crop productivity (Table 3). The maximum number of capsules (18.0) and seeds (160.5) per plant had the plants of the variety Vira at the application of $N_{90}P_{60}$ in the irrigated conditions. However, the highest weight of seeds per plant (1.28 g) with the 1000 seeds weight of 9.06 g was obtained from the plants of the variety Evryka at the application of $N_{90}P_{60}$. The maximum 1000 seeds weight both in the irrigated (8.94-9.06 g) and rain-fed (7.90-8.05 g) conditions was formed by the plants of the oil-seed flax variety Evryka. The varieties Vira and Orfei performed better in the non-irrigated cultivation conditions. More capsules (11.5) and seeds (102.2) per plant were formed at the variety Vira, and weight of seeds per plant was more at the variety Orfei (0.60 g) at the application of $N_{90}P_{60}$ at the expense of higher 1000 seeds weight - 6.35 g.

Table 3. Productivity of the varieties of oil-seed flax depending on the studied factors, average for 2016-2018

Humidification conditions	Variety	Mineral nutrition background	Number of capsules per plant	Number of seeds per plant	Weight of seeds per plant	1000 seeds weight
Irrigation	Evryka	Control ($N_0 P_0$)	10.9	92.6	0.81	8.94
		$N_{45} P_{60}$	13.9	120.6	1.06	9.10
		$N_{60} P_{60}$	16.0	138.7	1.23	9.15
		$N_{90} P_{60}$	17.0	147.8	1.28	9.06
	Orfei	Control ($N_0 P_0$)	10.9	95.7	0.66	7.00
		$N_{45} P_{60}$	13.5	119.9	0.82	7.04
		$N_{60} P_{60}$	15.7	140.5	0.98	7.05
		$N_{90} P_{60}$	16.4	146.3	1.04	7.18
	Vira	Control ($N_0 P_0$)	13.8	122.5	0.80	6.57
		$N_{45} P_{60}$	16.1	143.4	0.94	6.59
		$N_{60} P_{60}$	17.0	152.5	1.01	6.67
		$N_{90} P_{60}$	18.0	160.5	1.05	6.64
Rain-fed	Evryka	Control ($N_0 P_0$)	6.4	52.5	0.41	7.90
		$N_{45} P_{60}$	7.7	64.6	0.50	8.04
		$N_{60} P_{60}$	8.2	69.2	0.55	8.12
		$N_{90} P_{60}$	8.6	72.5	0.57	8.05
	Orfei	Control ($N_0 P_0$)	8.7	75.4	0.47	6.23
		$N_{45} P_{60}$	9.7	85.6	0.53	6.32
		$N_{60} P_{60}$	10.2	90.6	0.56	6.31
		$N_{90} P_{60}$	10.8	96.8	0.60	6.35
	Vira	Control ($N_0 P_0$)	9.2	81.9	0.48	5.91
		$N_{45} P_{60}$	10.6	94.7	0.54	5.84
		$N_{60} P_{60}$	10.8	98.0	0.56	5.87
		$N_{90} P_{60}$	11.5	102.2	0.59	5.96
LSD_{05}		Factor A	0.53	1.77	0.01	0.09
		Factor B	0.42	2.82	0.02	0.05
		Factor C	0.62	3.29	0.02	0.06

The results of correlation analysis allowed determination of relationships between the yield of oil-seed flax and the studied factors. There were determined close direct inter-connections between the yields of oil-seed flax varieties and the number of capsules, the number of seeds, the weight of seeds per plant, independently from the conditions of humidification and fertilizers' doses. The calculated coefficients pointed out that the productivity of oil-seed flax is determined by the number of capsules and seeds per plant, together with the weight of seeds per plant differently for the studied varieties: for Evryka $r = 0.82$, for Vira $r = 0.80$ in the irrigated conditions, and for Evryka $r = 0.91$, for Orfei $r = 0.95$ in the non-irrigated conditions, independently on the application of mineral fertilizers. The increase of yield caused the decrease in 1000 seeds weight of the variety Vira, that is proved by the coefficient of correlation value $r = 0.27$. This fact could be explained by the higher density of this variety in comparison to the other in the rain-fed conditions.

Yield formation is a difficult process that is genetically and environmentally determined. Obtaining high yields depends on the heat, moisture, and nutritive elements income to plants (Table 4).

The results of the study testify that the yield of oil-seed flax increases with the increase of the Nitrogen supply. The maximum yield of seeds of the variety Evryka (2.36 t ha^{-1}) was obtained in the irrigated conditions at the application of $N_{90}P_{60}$. The maximum yield of oil-seed flax in the rain-fed conditions was provided by the varieties Vira (1.47 t ha^{-1}) and Orfei (1.45 t ha^{-1}) at the application of $N_{90}P_{60}$. The differences between the variants with application of $N_{60}P_{60}$ and $N_{90}P_{60}$ were insignificant at $p < 0.05$.

The decrease in the dose of fertilizers application had a negative effect on the yield of the crop both in the irrigated and non-irrigated conditions, independently on the variety. The lowest yield in the study was obtained at the control (non-fertilized) variant with the variety Evryka under the rain-fed conditions of humidification - 0.99 t ha^{-1}. Application of $N_{90}P_{60}$ provided the yield at the level of 1.33 t ha^{-1} that is by 0.14 t ha^{-1} lower than of the variety Vira at the same conditions of cultivation.

Our study proved that the variety played significant role on the oil content in oil-seed flax seeds (Table 5).

The highest oil content of 47.4% was obtained at the oil-seed flax variety Vira in the irrigated conditions on the plots with the application rate of mineral fertilizers $N_{90}P_{60}$, when the yield of oil was 0.97 t ha^{-1}. In the rain-fed conditions this variety also provided the highest oil content of 46.0% with the yield of oil 0.57 t ha^1 under the application of $N_{60}P_{60}$.

The economic efficiency of the crop cultivation is represented in the Table 6.

Table 4. Yields of oil-seed flax varieties in dependence on the conditions of soil humidification and mineral nutrition background, average for 2016-2018

Humidification conditions (Factor A)	Variety (Factor B)	Mineral nutrition background (Factor C)	Yield of seeds, t ha^{-1}			Average yield of seeds for 2016-2018, t ha^{-1}
			2016	2017	2018	
Irrigation	Evryka	Control (N_0 P_0)	1.48	1.77	1.86	1.70
		N_{45} P_{60}	1.76	2.04	2.39	2.06
		N_{60} P_{60}	1.99	2.28	2.42	2.23
		N_{90} P_{60}	2.19	2.41	2.48	2.36
	Orfei	Control (N_0 P_0)	1.44	1.74	1.71	1.63
		N_{45} P_{60}	1.76	1.98	2.18	1.97
		N_{60} P_{60}	1.93	2.19	2.25	2.12
		N_{90} P_{60}	2.11	2.33	2.29	2.24
	Vira	Control (N_0 P_0)	1.43	1.83	1.69	1.65
		N_{45} P_{60}	1.78	2.24	2.18	2.06
		N_{60} P_{60}	1.94	2.37	2.20	2.17
		N_{90} P_{60}	2.13	2.49	2.27	2.29
Rain-fed	Evryka	Control (N_0 P_0)	1.25	1.02	0.71	0.99
		N_{45} P_{60}	1.56	1.19	0.84	1.20
		N_{60} P_{60}	1.74	1.27	0.86	1.29
		N_{90} P_{60}	1.88	1.33	0.79	1.33
	Orfei	Control (N_0 P_0)	1.29	1.15	0.73	1.06
		N_{45} P_{60}	1.58	1.31	0.91	1.27
		N_{60} P_{60}	1.76	1.39	0.96	1.37
		N_{90} P_{60}	1.92	1.48	0.96	1.45
	Vira	Control (N_0 P_0)	1.35	1.20	0.78	1.11
		N_{45} P_{60}	1.59	1.34	0.94	1.29
		N_{60} P_{60}	1.76	1.41	1.00	1.39
		N_{90} P_{60}	1.96	1.49	0.97	1.47
LSD_{05}		Factor A	0.04	0.03	0.10	0.06
		Factor B	0.02	0.02	0.10	0.09
		Factor C	0.01	0.02	0.11	0.08

Table 5. Effect of different doses of mineral fertilizers and soil humidification on the qualitative indices of oil-seed flax oil, average for 2016-2018

Humidification conditions (Factor A)	Variety (Factor B)	Mineral nutrition background (Factor C)	Oil content, %	Yield of oil, t ha^{-1}
Irrigation	Evryka	Control (N_0P_0)	43.3	0.66
		$N_{45}P_{60}$	43.9	0.80
		$N_{60}P_{60}$	44.8	0.89
		$N_{90}P_{60}$	45.0	0.94
	Orfei	Control (N_0P_0)	45.1	0.65
		$N_{45}P_{60}$	45.2	0.79
		$N_{60}P_{60}$	46.4	0.88
		$N_{90}P_{60}$	46.2	0.92
	Vira	Control (N_0P_0)	45.2	0.66
		$N_{45}P_{60}$	45.1	0.83
		$N_{60}P_{60}$	46.1	0.89
		$N_{90}P_{60}$	47.4	0.97
Rain-fed	Evryka	Control (N_0P_0)	43.5	0.38
		$N_{45}P_{60}$	43.2	0.46
		$N_{60}P_{60}$	43.7	0.51
		$N_{90}P_{60}$	43.6	0.52
	Orfei	Control (N_0P_0)	42.0	0.40
		$N_{45}P_{60}$	44.9	0.51
		$N_{60}P_{60}$	45.0	0.55
		$N_{90}P_{60}$	44.7	0.58
	Vira	Control (N_0P_0)	42.8	0.42
		$N_{45}P_{60}$	45.7	0.52
		$N_{60}P_{60}$	46.0	0.57
		$N_{90}P_{60}$	45.3	0.59
LSD_{05}		Factor A	0.30	0.065
		Factor B	0.20	0.025
		Factor C	0.27	0.017

Table 6. Economic efficiency of oil-seed flax cultivation depending on the variety, soil humidification and mineral nutrition, average for 2016-2018 (currency exchange rate applied in the calculations is 1 USD = 26.00 UAH)

Humidification conditions (Factor A)	Variety (Factor B)	Mineral nutrition background (Factor C)	Value of production, USD ha^{-1}	Expenditures, USD ha^{-1}	Profit, USD ha^{-1}	Profitability, %
Irrigation	Evryka	Control (N_0P_0)	784.62	250.35	534.27	213
		$N_{45}P_{60}$	950.77	307.12	643.65	210
		$N_{60}P_{60}$	1029.23	367.62	661.62	180
		$N_{90}P_{60}$	1089.23	389.19	700.04	180
	Orfei	Control (N_0P_0)	752.31	268.27	484.04	180
		$N_{45}P_{60}$	909.23	325.77	583.46	179
		$N_{60}P_{60}$	978.46	384.73	593.73	154
		$N_{90}P_{60}$	1033.85	410.12	623.73	152
	Vira	Control (N_0P_0)	761.54	260.19	501.35	193
		$N_{45}P_{60}$	950.77	317.69	633.08	199
		$N_{60}P_{60}$	1001.54	376.65	624.88	166
		$N_{90}P_{60}$	1056.92	402.04	654.88	163
Rain-fed	Evryka	Control (N_0P_0)	456.92	207.62	249.31	120
		$N_{45}P_{60}$	553.85	265.00	288.85	109
		$N_{60}P_{60}$	595.38	270.12	325.27	120
		$N_{90}P_{60}$	613.85	310.85	303.00	97
	Orfei	Control (N_0P_0)	489.23	209.38	279.85	134
		$N_{45}P_{60}$	586.15	266.77	319.38	120
		$N_{60}P_{60}$	632.31	275.73	356.58	129
		$N_{90}P_{60}$	669.23	312.65	356.58	114
	Vira	Control (N_0P_0)	512.31	201.31	311.00	154
		$N_{45}P_{60}$	595.38	258.73	336.65	130
		$N_{60}P_{60}$	641.54	267.69	373.85	140
		$N_{90}P_{60}$	678.46	307.08	371.38	121

The best performance in the irrigated conditions was provided by the variety Evryka, which formed the maximum yield of 2.36 t ha^{-1} with the highest profit of 700.04 USD ha^{-1} under the application of mineral fertilizers in the dose $N_{90}P_{60}$.

The profitability level was 180%. However, the best yield of oil was provided by the variety Vira (47.4% or 0.97 t ha^{-1}).

It was determined that the variety Vira is the best one for cultivation in the rain-fed conditions of the South of Ukraine. This variety provided the yield of 1.39 t ha^{-1} at the application of $N_{60}P_{60}$ with the highest profit (373.85 USD ha^{-1}) and profitability (140%). The oil content of this variety was 46.0%, and the yield of oil was 0.57 t ha^{-1}. At the same time, the control variant provided profitability of 154% with the yield of oil just 42.8%.

It was also discovered that cultivation of oil-seed flax in the irrigated conditions provided the increase of seed yield in average by 41% or 0.97 t ha^{-1}.

Evaluation of drought tolerance of the studied varieties of oil-seed flax was performed through the calculation of the corresponding indices that consider the level of yield losses in drought conditions in comparison to optimal conditions. These indices show both tolerance and susceptibility of the varieties to drought (Bouslama & Schapaugh, 1984; Rudik, 2013). The reaction of genotypes to drought was performed by the yielding index (Zinchenko et al., 2001). Mathematical processing and calculation of the above-mentioned indices significantly simplifies the process of drought tolerance assessment (Vus et al., 2017).

The main indices of drought tolerance, namely, mean productivity (MP) - 1.30, yield stability index (YSI) - 0.55, yielding index (YI) - 106, stress tolerance index (STI) - 0.47, revealed that the variety Vira was the best one among the studied varieties of oil-seed flax (Table 7).

Table 7. Comparison of the indices of drought tolerance of the studied varieties of oil-seed flax, average for 2016-2018

Variety	DSI	TOL	MP	YSI	YI	STI	GMP
Evryka	1.09	0.81	1.21	0.50	92	0.39	1.13
Orfei	0.98	0.75	1.27	0.54	102	0.44	1.21
Vira	0.96	0.75	1.30	0.55	106	0.47	1.24

Note: DSI - drought susceptability index; TOL - index of tolerance to drought; MP - mean productivity; YSI - yield stability index; YI - yielding index; STI - stress tolerance index; GMP - geometrically mean productivity.

Drought tolerance is an important quality of oil-seed flax, especially, in the modern conditions of the changes in regional and global climate (Lykhovyd, 2018) and modern challenges, which arose due to the lack of freshwater for irrigation needs (Vozhehova et al., 2018).

Multiple linear regression analysis revealed general tendency to the increase in oil-seed yield by 6.431 kg with the increase of irrigation rate by every 1 mm; by 4.548 kg per every 1 kg ha^{-1} of Nitrogen fertilizers; by 1.228 kg per every 1 kg ha^{-1} of Phosphorus fertilizers, respectively.

The results of ANOVA are represented in the Table 8, and the results of regression analysis are provided in the Table 9.

Table 8. ANOVA results for multiple regression analysis of oil-seed flax seed yield depending on humidification conditions and mineral fertilizers

	Degrees of freedom	Sum of squares	Mean squares	F	F significance
Regression	3	4.411	1.470	241.785	7.07×10^{-16}
Residuals	20	0.122	0.006		
Total	23	4.533			

Table 9. Regression statistics for the analysis of oil-seed flax yield depending on humidification conditions and mineral fertilizers

	Coefficients	Standard errors	t-statistics	P-value
Y-interception	0.970833	0.035594	27.27499	2.70×10^{-17}
Imput X1	0.006431	0.000265	24.23844	2.68×10^{-16}
Input X2	0.004548	0.000982	4.628643	0.000162
Input X3	0.001685	0.001228	1.371629	0.185372

Note: Y - yield, t ha^{-1}; Input X 1 - irrigation rate, mm; Input X 2 - Nitrogen fertilizer dose, kg ha^{-1}; Input X 3 - Phosphorus fertilizer dose, kg ha^{-1}.

Table 10. Approximation of the regression model of oil-seed falx yield depending on humidification conditions and mineral fertilizers

Input X1 (irrigation rate, mm)	Input X2 (Nitrogen dose, kg ha^{-1})	Input X3 (Phosphorus dose, kg ha^{-1})	True yields, t ha^{-1}	Modeled yields, t ha^{-1}	Residuals (modeled-true), t ha^{-1}
0	0	0	0.99	0.97	-0.02
0	45	60	1.20	1.28	0.08
0	60	60	1.29	1.34	0.05
0	90	60	1.33	1.48	0.15
0	0	0	1.06	0.97	-0.09
0	45	60	1.27	1.28	0.01
0	60	60	1.37	1.34	-0.03
0	90	60	1.45	1.48	0.03
0	0	0	1.11	0.97	-0.14
0	45	60	1.29	1.28	-0.01
0	60	60	1.39	1.34	-0.05
0	90	60	1.47	1.48	0.01
120	0	0	1.70	1.74	0.04
120	45	60	2.06	2.05	-0.01
120	60	60	2.23	2.12	-0.11
120	90	60	2.36	2.25	-0.11
120	0	0	1.63	1.74	0.11
120	45	60	1.97	2.05	0.08
120	60	60	2.12	2.12	0
120	90	60	2.24	2.25	0.01
120	0	0	1.65	1.74	0.09
120	45	60	2.06	2.05	-0.01
120	60	60	2.17	2.12	-0.05
120	90	60	2.29	2.25	-0.04

The equation of oil-seed yield depending on the studied factors looks like $Y = 0.970833 + 0.006431 \times X1 + 0.004548 \times X2 + 0.001685 \times X3$.

The values of regression statistics, namely: multiple coefficient of regression R - 0.9865; square coefficient of regression R^2 - 0.9732; normalized R^2 - 0.9691; standard error - 0.0779 testify about high accuracy of the developed model, which was also proved by its approximation, showing a slight amplitude of residuals not exceeding 0.15 t ha^{-1} with average error of modeling averaging to 3.71% (Table 10).

The results of regression analysis explain peculiarities of the crop productivity formation and point out a great importance of irrigation for oil-seed flax production in the South of Ukraine. It was also determined that the least effect on the crop yield is associated with application of Phosphorus fertilizers, and

Nitrogen had a moderate level of the impact on the yield.

Nowadays, linseed flax is cultivated in 64 countries of the world, and the areas under the crop are increasing. And yield of the crop is highly different in different regions of the world, and even through the European countries (D'Antuono & Rossini, 2006). One of the main oil-seed and fiber flax producers is the United Kingdom, where the crop is cultivated both as a fiber and oil crop to provide the maximum economic efficiency and benefits of the production (Foster et al., 1998). It was suggested that oil-seed flax has a potential to become the third main oil crop in the world production, standing in the one row with rape and sunflower (Diepenbrock, 2001). And European countries in general and Ukraine are one of the most important areas of the world linseed production. That is why it is so important to conduct comprehensive investigation regarding the crop productivity.

It was evaluated that the maximum potential yield of oil-seed flax seeds at the level of 4.5-5.0 t ha^{-1} could be obtained under the following combination of cultivation conditions: the density of plants 6-7 million of plants ha^{-1}, number of capsules per stem 15-22, number of seeds per capsule 8-9, 1000 seeds weight 5.5-6.0 g, dry weight herbage biomass yield of 9.0-10.0 t ha^{-1} (Candrakova & Bakula, 2001). It is believed that plant density and number of capsules per plant have the greatest effect on the yield of oil-seed flax (Zajac et al., 2012). This presumption was proved by our results that testified about the fact that the yield of seeds had close connection with the number of capsules and seeds per plant, together with the weight of seeds per plant, according to the results of correlation analysis. Besides, the study conducted in Turkey reports about the same tendency: the highest close direct positive influence on the yield of linseed was provided by the number of capsules per plant with *r* value of 0.797 (Copur et al., 2006). However, earlier work by Zajac et al. (2005) reports that linseed yield is dependent mostly on the growing season conditions and features of the cultivated genotype of the crop. The study conducted by Al-Doori (2012) reports about significant dependence of linseed yield on the date of sowing and variety (genotype). It is evident that right chosen varieties for concrete climatic conditions provide the best productivity that was also certified by the results of our study, where the variety Evryka was the number one in the irrigated conditions, and the variety Vira - in the rain-fed conditions, respectively. Additionally, the comprehensive study in regard to the response of oil-seed flax on different technological options discovered that the seed yield is significantly affected by the dose of Nitrogen applied (the best variant was with 100% supply of the crop with this element accordingly to the results of the soil test) and plant density (should be higher than 300 plants m^{-2}) with no significant effect of sowing date on the crop productivity (Lafond et al., 2008). It was stated that the optimum date of sowing is highly dependent on the latitude of the crop cultivation with the general tendency of better sowing later in the North, and earlier sowing in the South. The studied by Lafond et al. (2008) cultivars did not show significant difference in the yield. But Gallardo et al. (2014) agree with us, namely, they also report about significant dependence of seed yield and linseed oil quality on the variety. Besides, they proved that delayed sowing of the crop in Argentina should not be practiced.

The results of Berti et al. (2009) agree with ours in the point of the reaction of oil-seed flax to mineral fertilization with Nitrogen and Phosphorus. They also proved significant increase in the seed yield with the increase of Nitrogen supply; however, they used enormously high rates of this nutritive element of 200 kg ha^{-1} that is not environmentally friendly in our opinion. The results of another study discovered that the application of $N_{60}P_{40}S_{30}$ significantly increased the yield of flax on the loam and sandy loam soils (Aulakh et al., 1989). The deficiency of Nitrogen results in considerable decrease of oil-seed flax yield and yield components, therefore, artificial Nitrogen supply is an irreplaceable element of the crop cultivation (Hocking & Pinkerton, 1991).

Besides the factors studied in current research, there are a number of other important technological factors of oil-seed flax productivity regulation, namely: sowing depth and preparation of the seedbed (Couture et al., 2004); row spacing of the crop (Kocjan Acko &

Trdan, 2008); fertilization with macro (NPK) and micronutrients (Berti et al., 2009). Thereby, further scientific researches are required to create a comprehensive image of oil-seed flax response to different agricultural treatments in different environmental conditions, considering soil and climate factor of the crop productivity (Dillman, 1943).

CONCLUSIONS

The maximum yields of seeds were provided by the oil-seed flax variety Evryka (2.36 t ha^{-1}) in the irrigated conditions, and the variety Vira (1.47 t ha^{-1}) in the non-irrigated conditions at the application of $N_{90}P_{60}$. The best yield of oil was provided by the variety Vira (0.97 t ha^{-1} and 0.57 t ha^{-1} with the application of $N_{60}P_{60}$ in the irrigated and non-irrigated conditions, respectively). The variety Vira has the highest drought tolerance among the studied varieties of oil-seed flax. By the means of statistical yield analysis, it was revealed that the strongest effect on the crop yield is caused by irrigation, and the least - by Phosphorus fertilization. Mineral nutrition must be adjusted with accordance to the field fertility level with taking into account humidification conditions. The main regularity is: Nitrogen supply of the crop should be strengthened at the irrigated conditions, and the crop should not be over-fertilized with Nitrogen in the rain-fed conditions, especially, if the genotype of the cultivated variety is not drought tolerant enough.

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PHOTOSYNTHETIC ACTIVITY AND PRODUCTIVITY OF SUNFLOWER HYBRIDS IN ORGANIC AND TRADITIONAL CULTIVATION TECHNOLOGIES

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Abstract

The scientific paper presents the experimental data on the impact of different cultivation technologies on the indexes of assimilation surface area, chlorophyll content, its fractional composition (fractions "A" and "B") and the yields of sunflower hybrids under conditions of the Southern Ukrainian Steppe. The research was conducted in 2018-2019 on dark-chestnut soil in the semi-arid conditions. The research scheme consisted of the following factors and their variants: sunflower hybrids (PR64F66, Tunca); cultivation technologies (intensive; organic). The research results proved that the best conditions for the formation of the largest area of assimilation apparatus by sunflower plants were created in the organic technology (treatment of soil + seeds + plants with organic preparations during the growing season) when growing the hybrid Tunca: at the stage of 3 pairs of true leaves - 2.9; the formation of heads - 27.1; flowering - 37.1 and milky ripeness - 29.0 thousand m^2/ha. Under the same conditions there was maximum amount of chlorophyll (9.71 mg per 1 g of dry weight) and enzymes. Sunflower cultivation under the organic technology caused the formation of maximum yield (PR64F66 F_1 - 2.42 and Tunca F_1 - 2.41 t/ha), realization of biological productivity (90.9 and 90.7%) and fat content (48.7 and 49.8%).

***Key words**: chlorophyll, intensive and organic technologies of cultivation, leaf surface area, sunflower, yield.*

INTRODUCTION

Increasingly progressing expansion of marginal crops is a feature of the current agro-phytocenosis without any exceptions, indicating that agricultural producers bring economic indexes of farming to the foreground, while ecological aspects are either left without attention, or, at the best, considered by the leftover principle (Andrienko, 2011). Therefore, finding a solution to this problem (reduction of pressing on agro-landscapes and simultaneous maintenance of efficiency indexes of economic activity) is a promising and topical task for researchers (Tkalich et al., 2014; Lavrenko et al., 2019; Lavrenko & Maksimov, 2016).
Currently sunflower is not only a leading oil crop of the domestic agrocenosis, but it often takes a major place in the total structure of the sown areas of an ordinary agricultural enterprise. In 2019 the gross yield of sunflower in Ukraine made 14.5 million tons with the average yield of 2.6 tons per hectare. The complex of reasons causing this phenomenon includes objective and subjective factors of economic, social and technological nature, and the issues of the optimal amount of the crop in the field crop rotations are among the most discussed ones for both scientists and practitioners of agricultural production (Basali & Dobrovolsky, 2015).
However, more and more representatives of the scientific community and agricultural producers agree that the current state of the problem with excessive expansion of sunflower and systematic deviation from the scientifically substantiated zonal technologies require transition to modern technologies (Tkalich et al., 2014).
Biologization of sunflower production is a main method to solve the current problem in the market of agricultural products (Maslak, 2015; Giles, 2004; Leifeld & Fuhrer, 2010). It can be solved in a complex way taking into consideration both biotic and abiotic factors of agrocenosis (Basali et al., 2016).
According to the data obtained by the scientists, when cultivating sunflower by organic technologies it is important to follow

all the requirements that can be checked by means of δ15N or other markers (Joergensen et al., 2019; Camin et al., 2011).

Sustainable agricultural practices that enhance soil fertility and increase its capacity for carbon sequestration are increasingly needed (Abdallah et al., 2019)

It is also known that application of organic fertilizers increases the content of linoleic and oleic acids, improving oil quality. The use of them increases sunflower drought-resistance (Jami et al., 2019).

The application of manure doses of 12.5 and 15% of manure on a weight basis improved the early growth of the sunflower, with significant increases in the growth variables (Barros et al., 2019).

It is widely known that microbial biomass of soil is one of the main factors in determining soil fertility (Fließbach & Mäder, 2000; Tu et al., 2006; Lykhovyd et al., 2017). Organic agriculture ensures higher development of microbial community, than traditional agriculture (average 29.1 and 22.5 kg DNA ha^{-1}, respectively) (Verdi et al., 2019).

Currently the productivity of sunflower seeds in traditional systems of agriculture is higher than that of organic systems. Scientists think that it is a result of decreasing the amount of available forms of nitrogen of organic fertilizers (Verdi et al., 2019; Mäder et al., 2002).

The goal of our field study was to discover the best agro-technological options for sunflower production (including hybrids, cultivation technologies etc.) in the European semi-arid climatic zone of southern Ukraine to obtain the highest grain yield of the crop, quantitative and qualitative indexes of the sunflower leaf apparatus.

MATERIALS AND METHODS

Field trials on the sunflower cultivation technology were conducted during 2018-2019 in the non-irrigated lands of the Farm "Vera" in Hola Prystan district of Kherson region. The experimental plots were located at the latitude 46°20'16.11"N, longitude 32°17'31.38"E, and were elevated to 9 m above the sea level. The field experiments were conducted in four replications by using the split plot design method. The study was dedicated to evaluation of the following cultivation technology elements:

- A - sunflower hybrid: A1 - sunflower hybrid PR64F66 F_1 (bred by Pioneer); A2 - sunflower hybrid Tunca F_1 (bred by Limagrain);
- B - cultivation technology: B1 - intensive; B2 - treatment of soil with organic preparations; B3 - treatment of seeds with organic preparations; B4 - treatment of plants with organic preparations during the growing season; B5 - treatment of soil + seeds + plants with organic preparations during the growing season.

The climate of the experiment zone is characterized as comparatively dry and hot, with the average annual air temperature of 9.8ºC that has a tendency to further increase (Lykhovyd et al., 2018). According to the data provided by Kherson regional hydro-meteorological center, the total rainfall amounts in the zone average to 441 mm, while evapotranspiration reaches 1000 mm. The main meteorological indexes for the period of sunflower vegetation in the experiments are given in Table 1.

Table 1. Meteorological indexes during the period of sunflower vegetation in the field experiments

2018			2019			Long-term data			Months
AT, ºC	PA, mm	RH, %	AT, ºC	PA, mm	RH, %	AT, ºC	PA, mm	RH, %	
3.6	71.9	77	6.6	46.2	81	3.3	26.0	79	March
10.6	8.2	60	11.4	62.3	67	10.0	33.0	70	April
15.3	47.1	61	14.9	29.7	70	16.0	42.0	66	May
21.3	62.0	56	21.1	38.1	64	19.9	45.0	65	June
22.5	5.9	60	22.8	137.0	65	21.9	49.0	63	July
24.2	39.5	59	24.3	0.6	67	21.3	38.0	62	August
18.0	19.5	62	16.3	83.0	70	16.4	40.0	68	September

The soil of the experimental plots was represented by the dark-chestnut middle-loamy middle-saline soil with the humus content of 2.34-2.60%. The content of mobile forms of the elements of mineral nutrition: nitrogen - 1.7-2.0; phosphorus - 4.9-6.5; potassium - 28-36 mg-eq in 100 g of soil, pH - 6.9-7.2. The soil has moderate natural fertility, which mainly depends on nitrogen content.

The sunflower cultivation technology in the experiments was based on the generally accepted recommendations for the crop cultivation in the South of Ukraine. The experiments used the medium-early simple sunflower hybrids of a linoleic type PR64F66 from Pioneer and Tunca F_1 from Limagrain.

The hybrid PR64F66 is resistant to the sunflower broomrape (*Orobanche cumana*) of 7 races (A-G), resistant to lodging (8 points), resistant to phomosis, phomopsis, false powdery mildew (7 points), white head rot (6 points), white stem rot (6 points) and it also has excellent drought-resistance (9 points). The average duration of the growing season is 110-115 days, the yield potential is 5.5 tons per hectare, the head is convex and oil content (6 points) is medium.

The hybrid Tunca is resistant to the sunflower broomrape of 7 races (A-G). The average diameter of the head is 15.9 cm, the average weight of 1000 seeds is 73 g and the height of the plants is an average of 1.5 m. It is distinguished by its plasticity to the growing conditions of the soil. It is resistant to drought, cold and lodging, that affects its stability in various climatic conditions, and on soils of different quality. It is capable of sustaining steady yields. It is tolerant to white head rot, has immunity to stressful conditions and genetic resistance to various diseases. The hybrid is characterized by resistance to such pathogens as *Phomopsis*, *Sclerotinia*, *Macrophomina*, *Rhizopus* and *Phoma*.

The previous crop was winter wheat. The soil was prepared by carrying out double disking at the depths of 6-8 and 10-12 cm. In 14 days after the last disking, the stubble was plowed to the depth of 25-27 cm. In the areas with the intensive cultivation technology, mineral fertilizers were applied at the basic tillage at the dose of P_{30} (in the form of granular super phosphate). In the spring, early spring harrowing was conducted to further level the soil. 14 days after harrowing, weeds were cultivated at the depth of 8-10 cm after weeding. The pre-sowing cultivation was carried out at the depth of 4-6 cm, under which the fertilizer was applied at the dose of $N_{30}P_{20}$ (in the form of ammonium nitrate and double super phosphate). In the areas where the organic cultivation technology was implemented, no fertilizer was used. Sunflower seeds in the variant of the traditional cultivation technology were inlaid with the mixture Cruiser 350 FS (the active substances include active ingredient thiamethoxam, 350 grams per liter) + Maxim (the active substances include fludioxonil, 25 grams per liter), seeds that were sown in the organic areas were treated with the preparation "Gilea-Start"® of 0.7 liter per ton (the active substances include chelate complex Mg, Fe, Co, Mo, Zn, Cu + EDTA).

Sunflower sowing was performed with the John Deere 7000 seeder when the soil temperature at the depths of 10 cm was 8-10°C, which corresponded to 2nd of April in 2018 and 11th of April in 2019. After sowing, the crops were rolled.

Amistar Extra fungicide at the rate of 1.0 liter per hectare (the active substances include azoxystrobin, 200 grams per liter + cyproconazole, 80 grams per liter) was used to protect the plants against diseases in the areas of the traditional cultivation during the vegetation. The organic technology used the organic bacterial fertilizer Gilea-Oliinyi, at the rate of 1.5 liter per hectare (the active substances include chelate complexes of macro, meso and trace elements "Gilea"® - Mg, Fe, Co, Mo, Zn, Cu, B, Mg + EDTA). Prior to the emergence of seedlings, the harrowing of the soil was carried out. During the growing season row spacing was cultivated at the depths of 5-7 and 6-8 cm.

Sunflower seeds were harvested using the continuous method from all the variants of the experiment at the full ripeness stage with the CLAAS Lexion combine. The harvesting took place on 29th of August in 2018 and 24th of August in 2019. The data obtained resulted in basic moisture (7%) and 100% purity.

The leaf surface area index and architectonics of a leaf blade were examined with the method of express-scanning, the content of a green pigment and its fractional composition was investigated with a photometric colorimetric method and spirit extract, the content and fractional content of ferments - with a photometric colorimetric method and acetone extract with hydrogen peroxide with further photoseparation. The experimental data of sunflower were processed by the standard procedure of ANOVA within MS Excel software. Significance of the differences was proved for the reliability level of 95% (LSD_{05}).

RESULTS AND DISCUSSIONS

The results of the study are given in Tables 2, 3, 4. The organic technology of growing sunflower hybrids was characterized by more favorable conditions for the formation of the assimilation apparatus: the total leaf surface area index and the index of foliage clumping of agro-phytocenosis by all the stages of the crop development were higher by 5.5-31% when compared to the plots where the intensive cultivation technology was implemented.

By the results of our research, the intensity of the formation of the assimilation apparatus area of both sunflower hybrids and its absolute value were higher in the organic technology of growing the crop.

The variant of the complex organic technology was characterized by the most essential impact on the index of the crop foliage clumping according to the results of our research. For instance, by the variant of the hybrid PR64F66 this index was 3.69 against 3.15 at the sunflower flowering stage on the average for the years of the research in the intensive cultivation technology; by the variant of the hybrid Tunca the assimilation apparatus area exceeded the crop sown area 3.71 times at the background of the complex organic cultivation technology against 3.22 in the traditional cultivation technology. At the same time we identified more essential dynamics of the loss of the crop assimilation apparatus at the final stages of the crop ontogenesis at the background of implementing the intensive cultivation technology.

Not only a green pigment content in the crop parenchyma is a very important index, but its fractional composition is also significant. With regard to this, the most technological measures are considered in terms of their impact on the content of the chlorophyll fraction “A” as the one that is the most essential in the formation of organic substance by a plant organism. (Table 3). The analysis of the experimental data given above allows drawing a conclusion that in the organic technology of growing sunflower more favorable conditions were created for the formation of a green pigment, the content of the physiologically active fraction “A” in it and the ferments responsible for antioxidant properties and oxygen exchange in the plant cells, resulting in the drought-resistance and heat tolerance of a plant organism in the years of the research.

The architectonics of the leaf blade and the plant general habitus on the test plots were less than the corresponding indexes on the plots where the elements of the organic technology were implemented, causing the change of the leaf blade (a more elongated and thinner blade of a light-green color, a more elongated plant with a longer distance between the levels of leaves). This variant of the technology of growing both hybrids ensures the maximum content of a green pigment in the crop leaves, its optimal fractional composition, and also the content of ferments maintaining the traffic of oxygen to the plant tissues under stressful conditions by the hydro-chemical coefficient (peroxidase and catalase).

The highest productivity of sunflower seeds was found to be 3.8% for growing the hybrid PR64F66 compared to the hybrid Tunca F1. It was also shown that growing crops in the organic technology contributed to the formation of seed yields higher by 10.3% when compared to the intensive technology.

Similar results were observed in terms of realization of biological productivity of the crop. The biological yield of the sunflower hybrid PR64F66 ranged from 87.4 to 90.9%, which is 3.6% more than the hybrid Tunca. The use of the organic technology for the sunflower cultivation compared to the intensive technology increased the biological yield from 80.5-87.4 to 90.7-90.9%.

The fat content of sunflower seeds was the highest for the cultivation of Tunca F1 - 48.6-49.8%, which is 0.8 less than that of the hybrid PR64F66. The use of the organic technology also contributed to the accumulation of fatty oil in the seeds up to 48.7-49.8%, which is higher by 0.9% when compared to the intensive (traditional) technology.

Table 2. Dynamics of the formation of the leaf area of the sunflower hybrids depending on the cultivation technology. thousand m^2 per ha (average for the period 2018-2019)

Sunflower hybrid	Cultivation technology	Crop development stages			
		3 pairs of true leaves	head formation	flowering	milky-wax ripeness
A1 (PR64F66 F_1)	B1	2.2	20.0	31.5	22.4
	B2	2.4	22.2	32.9	26.8
	B3	2.3	22.4	33.8	27.2
	B4	2.6	22.8	35.0	27.0
	B5	2.8	23.9	36.9	28.1
A2 (Tunca F_1)	B1	2.4	21.7	32.2	23.3
	B2	2.4	21.5	33.0	27.0
	B3	2.5	25.2	32.7	26.7
	B4	2.7	26.0	34.4	29.2
	B5	2.9	27.1	37.1	29.0
LSD_{05} for factors	A	0.17	0.54	0.86	0.57
	B	0.11	0.71	1.14	0.43
	AB	0.19	0.88	1.32	0.77

Note: All the differences between the studied variants are significant.

Table 3. Pigment and enzyme composition of the spongy parenchyma of sunflower hybrid leaves depending on the cultivation technology (average for the period 2018-2019)

Sunflower hybrid	Cultivation technology	Chlorophyll			Enzymes	
		total content, mg per 1 g dry weight	fraction "A",%	fraction "B",%	peroxidase, conventional unit per 1 g of wet weight	catalase, conventional unit per 1 g of wet weight
A1 (PR64F66 F_1)	B1	5.29	60.2	39.8	6.36	1524
	B2	5.88	64.2	35.8	6.39	1726
	B3	6.39	62.9	37.1	6.51	1752
	B4	8.11	70.1	29.9	6.57	1754
	B5	9.02	73.3	26.7	6.88	1789
A2 (Tunca F_1)	B1	6.23	74.0	26.0	6.43	1549
	B2	6.50	75.1	24.9	6.49	1622
	B3	6.37	75.8	24.2	6.61	1646
	B4	8.28	78.4	21.6	6.72	1690
	B5	9.71	79.9	20.1	6.82	1695
LSD_{05} for factors	A	0.79	9.33	12.2	0.05	13.30
	B	0.43	1.68	3.03	0.09	18.07
	AB	0.85	10.02	12.77	1.05	22.12

Note: All the differences between the studied variants are significant.

Table 4. Seed yield of sunflower hybrids and fat content depending on the cultivation technology (average for the period 2018-2019)

Sunflower hybrid	Cultivation technology	Yield, t per ha	Realization of biological productivity, %	Fat content, %
A1 (PR64F66 F_1)	B1	2.27	87.4	48.1
	B5	2.42	90.9	48.7
A2 (Tunca F_1)	B1	2.11	80.5	48.6
	B5	2.41	90.7	49.8
LSD_{05} for factors	A	0.07		
	B	0.12		
	AB	0.21		

Note: All the differences between the studied variants are significant.

The best conditions for the formation of the largest area of assimilation apparatus of sunflower plants were created in the organic technology (treatment of soil + seeds + plants with organic preparations during the growing season) when growing the hybrid Tunca: at the stage of 3 pairs of true leaves - 2.9; the formation of heads - 27.1; flowering - 37.1 and milky ripeness - 29.0 thousand m^2/ha. Under the same conditions there was maximum amount of chlorophyll (9.71 mg per 1 g of dry weight) and enzymes. Sunflower cultivation in the organic technology caused the formation of the maximum yield (PR64F66 F_1 - 2.42 and Tunca F_1 - 2.41 t/ha), realization of biological productivity (90.9 and 90.7%) and fat content (48.7 and 49.8%).

The previous experimental research proved that the prevailing majority of scientists prefer fragmentary examination of individual elements of biologization of sunflower cultivation technologies (application of bio-fungicides and organic fertilizers, minimization of application or flat refusal to use some types of mineral fertilizers, reduction of their doses and rates, revision of the methods of applying them, involvement of biologically active substances of organic nature to the cultivation technology etc.) (Kadyrov & Silin, 2015). Another group of scientists follow a different concept: they declare scientifically substantiated amount of the crop in agro-phytocenosis (15-16%) without any principal revision of the zonal cultivation technology towards the implementation of the elements of biologization into it (Zhuk et al., 2011).

The issue of the research on the content of ferments directly causing the resistance of a plant organism to soil and, first of all, to air droughts - peroxidase and catalase - in spongy leaf parenchyma has been left without any attention in scientific periodicals and dissertation theses.

According to the results of our research, the intensity of the formation of the assimilation apparatus area of both sunflower hybrids and its absolute value were higher in the organic technology of the crop cultivation (Table 2).

According to the research results, the most essential impact on the index of the crop foliage clumping was characteristic of the variant B5 (treatment of soil + seeds + plants with organic preparations during the growing season). When growing the hybrid PR64F66, this index was 3.69 at the stage of sunflower flowering on the average for the years of the research against 3.15 in the intensive cultivation technology. The assimilation apparatus area of the hybrid Tunca exceeded 3.71 times the crop sown area in the technology under study B5 when compared to B1, where the corresponding index was 3.22.

We noticed more considerable loss of sunflower photosynthetic assimilation apparatus at the stage of full ripeness in the intensive cultivation technology.

Examining not only the content of chlorophyll in the crop parenchyma, but also its fractional composition is a modern trend of agronomy science. Currently the issue of fractional composition of chlorophyll and enzymes depending on different types of sunflower cultivation technology has not been thoroughly investigated. And the majority of technological measures are examined in the aspect of their impacts on the content of the fraction of chlorophyll “A” as the one that is the most essential in the formation of organic substance by a plant organism (Table 3).

Our research shows that in the organic technology of sunflower cultivation (B1-B5) for the years of the research there were more favorable conditions for the formation of a green pigment, the content of physiologically active fraction “A” in it and the ferments responsible for antioxidant properties and oxygen exchange in the plant cells, and it resulted in drought- and heat-resistance of a plant organism.

The previous research conducted on many agricultural crops proved inhibiting impact of synthetic pesticides on the amount and activity of micro-biota available in the soil (Fadeev, 2016: Lavrenko, 2007). Application of high rates of mineral fertilizers and a large number of chemical substances essentially reduce micro-biota in the soil and affect further reduction of the activeness of organic substance degradation, reduction of soil fertility and quality and also the crop productivity (Baghbani-Arani et al., 2017; Bajgai et al., 2015; Fliebbach et al., 2007; Leskovar et al., 2018; Lima et al., 2009; Ushkarenko et al., 2008).

An average of two years of the research proved that the yield of the conditioned seeds of the hybrid PR64F66 exceeded that of Tunca according to the variant of the traditional cultivation technology by 0.16 t, while no significant difference was recorded in the organic cultivation technology. At the same time, the variant of the organic cultivation technology was characterized by a significantly higher level of realization of biological yield (90.8% against 83.9%) and the index of fat content in the seed (49.3% against 48.3% respectively).

CONCLUSIONS

The best conditions for the formation of the largest area of assimilation apparatus by sunflower plants were created in the organic technology (treatment of soil + seeds + plants with organic preparations during the growing season) when growing the hybrid Tunca: at the stage of 3 pairs of true leaves - 2.9; the formation of heads - 27.1; flowering - 37.1 and milky ripeness - 29.0 thousand m^2/ha. Under the same conditions there was maximum amount of chlorophyll (9.71 mg per 1 g of dry weight) and enzymes.

Sunflower cultivation under the organic technology caused the formation of maximum yield (PR64F66 F_1 - 2.42 and Tunca F_1 - 2.41 t/ha), realization of biological productivity (90.9 and 90.7%) and fat content (48.7 and 49.8%).

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