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CEREALS AS ENERGY SOURCES IN THE FUNCTION OF CIRCULAR ECONOMY

SUMMARY

The circular economy is an approach that integrates the economy, the waste management system and protects the environment. The goal of the circular economy is to optimize the existing system and increase welfare. The growth trend of alternative fuels is evident in the world from year to year, thanks to the development of new technologies for processing biological waste into energy sources. Commercial production needs to be economically and environmentally friendly so that biofuels from renewable sources are an adequate substitute for fossil fuels. In this study, two winter grains, wheat and rye, were examined, during 2018/2019 and 2019/2020, in order to obtain biogas from plant biomass. The results showed that wheat had a statistically significantly higher production of biomass and biogas compared to rye. Based on the analysis of variance, it can be concluded that there are highly significant differences in biomass yield in regard to the genotype ($F_{\text{exp}}=937.75^{**}$) and years ($F_{\text{exp}}=28.07^{**}$) of investigation. There is a strong positive correlation between biomass and biogas yields.

Keywords: wheat and rye, biomass, biofuels, correlations, economic and environmental profitability

INTRODUCTION

Wheat (*Triticum vulgare* L.) and rye (*Secale cereale* L.) are species that represent one of the most important and earliest domesticated grains in the Old World (Glamočlija et al., 2015). Wheat was grown in the world in 2019 on 216,000,000 ha, with an average grain yield of 3.55 t ha⁻¹ and a total production of 766,000,000 t, and rye on 4,213,392 ha with average grain yields 3.04 t ha⁻¹ and with production of 12.801.441 t (Faostat, 2021). On the territory of the

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Republic of Serbia, wheat is grown on an area of about 600,000 ha, with an average yield of 4.4 t ha⁻¹ and production of 2,535.00 t, while rye is grown on average on an area of about 6,000 ha with an average yield of 2.6 t ha⁻¹ and production of 13.000 t (RZS, 2021). The circular economy represents a new approach that integrates the economy and the waste management system, opens up innovative and more efficient ways of production with global efforts for sustainable development. The circular economy model is in stark contrast to the dominant linear economy that promotes the concept of production called “take (from nature), make (in the production process), use and discard (waste).” The circular economy promotes competitiveness, innovation, protects living environment and at the same time contributes to economic growth. It is based on several principles: that products be designed to be reusable after use, and that the energy used in production comes from renewable sources, all with the aim of reducing dependence on resources. Thanks to the development of new technologies for processing bio-waste into energy, the growth rate of the use of alternative fuels is growing significantly (Popovic et al., 2020a; 2020b; 2020c; Ikanovic et al., 2020; Milanovic et al., 2020; Rakascan et al., 2021). According to estimates by energy experts, in highly developed countries it is about 15% per year. Rakascan et al. (2019a) point out that the cultivation of energy crops helps the development of rural areas (production of raw materials), but also industrial production, which provides the possibility of greater employment in primary crop production and biofuel production. The term biogas means gas produced in anaerobic fermenters and controlled conditions, i.e. in biogas plants. A mixture of gases, whose volume consists of about two thirds of methane and one third of carbon dioxide, is called biogas. By aerobic fermentation, organic biomass is decomposed to carbon dioxide, while methane is obtained by anaerobic means. Methane is a fuel gas, and the goal is precisely its production and use as an energy source (Glamoclija et al. 2015; Popovic et al., 2020b).

Straw, as an agricultural by-product, represents dry grain stalks, after the separation of grain and chaff, and makes up half of the grain yield. After harvest, straw is often burned in the fields. This is the most irrational way of using straw because burning burns all the organic substance that the useful soil fauna would decompose into forms that future crops can use for their needs. Straw burning should be decided only if the wheat was infected with the pathogenic fungus *Fusarium* sp. and *Erysiphe* sp. (Glamoclija et al. 2015). In the case of true cereal varieties, which have lower stalks and are less pruned, the share of harvest residues in the total yield is 40-50%. With a grain yield of 5,000 kg ha⁻¹, 3,500-4,500 kg ha⁻¹ straw can be obtained. According to the results of the research, which are stated by British authors, if the straw of cereals, grown only in the area of the eastern part of the Midlands, were used to obtain biofuels, the amount of obtained energy would cover about 1.5% of British consumption. However, the views of local farmers are explicit, and they insist that these secondary products be returned to the land by plowing or as manure, which has a far greater importance on soil fertility and further plant production. Finding the optimal

solution for the use of cereal straw should be the subject of further research, as Copeland and Turley (2008) point out. Straw can also be used to obtain liquid biofuels (ethanol) because it has large amounts of carbohydrates. Today, in addition to the requirements for fuel quality, there are increasing requirements for low exhaust emissions of toxic gases and obtaining fuel from renewable energy sources.

The aim is to produce new fuels on new technologies that offer significant potential in improving air quality and reducing gas emissions, all with the aim of protecting the environment and the impact on global climate change. However, the complete penetration of new technologies on the market is a long process, and some of the new technologies are still in the development phase and require significant investments until final application (Lakic et al., 2018; Ikanovic et al., 2018; Jankovic et al., 2017; 2019). The situation is similar in the field of fuel application technologies. All these circumstances force the world factors in the automotive industry and energy in general, to develop new types of fuels from renewable sources from biomass of agriculture and agricultural crops.

In the fight against current problems, last year, within the 21st conference on climate change held in Paris, a new global agreement was adopted, which has a much more ambitious plan than the previous one. 196 member states, including the Republic of Serbia, have committed themselves to the obligatory reduction of greenhouse gases, and the allowed rise in temperature to 1.5 ° C starting in 2020. The obligation of our country is to reduce emissions by 9.8% by 2030 compared to 1990. As an economically much weaker country, compared to developed European countries, Serbia lags far behind in terms of production and application of clean energy, although it cannot be disputed the fact that we have natural resources that should be used for the purpose of obtaining energy as they point out (Jankovic et al., 2017). The essence of converting harvest and all other plant residues into biofuels is not only obtaining as much energy as possible, but also applying a sustainable method of production from which everyone will benefit.

The aim of this study was to examine the possibility of using wheat and rye biomass as an energy source in the process of obtaining biogas.

MATERIAL AND METHODS

Experimental design

During 2018/2019 and 2019/2020, a field micro-survey was set up according to a random block system in 10 repetitions with the size of the basic plots of 10 m² (5 m x 2 m) in Dolovo (44° 54' N, 20° 52' E, 101 m a. s. l.) near Pancevo, on chernozem type land. The subject of the research were two winter cereals: wheat, variety Ilina (created at the Field and Vegetable Crops, Novi Sad) and rye, variety Tayo (KWS) and the examined parameters are productivity parameters: biomass yield and biogas yield. During the vegetation season, standard technological practices for growing winter cereals were applied. For the analysis of productive traits, samples were taken from the cut biomass at the stage of technological maturity. The technology of obtaining biogas from biomass was

performed in a biodigester, where the biomass was previously ground and inserted into a biodigester where fermentation and biogas production are performed. Since its inception *Biogas Energy* (company) has been involved in research into the possibilities of improving the supply of biomass for its needs in an environmentally, economically and energy sustainable way. Biogas yield was determined by analysis of wheat and rye silage in the laboratory of the Technical Faculty in Novi Sad and calculated on cubic meters per ton.

Statistical analysis

The analysis of the obtained experimental data was performed through analytical statistics with the help of the statistical package STATISTICA 12 for Windows (*StatSoft*). The obtained results are shown in the table. The significance of differences in mean values of the treatments was tested by the LSD test. Relative dependence was defined through correlation analysis (Pearson's correlation coefficient), and the coefficients that were obtained were tested at the 5% and 1% levels of significance.

RESULTS AND DISCUSSION

Meteorological conditions

Climatic parameters were obtained from the meteorological station in Pancevo the average monthly temperatures during the research years. City Pancevo is located in the Autonomous Province of Vojvodina, in the Republic of Serbia on the banks of rivers Tamis and Danube, in the southern part of Banat in a fertile valley at an altitude of 77 m (44° 54' 02" N; 20° 52' 23" E, 101 m asl). The South Banat administrative district extends in the northern part of Serbia, ie in the south-eastern part of the northern Serbian province, Vojvodina, (Pictures 1a, 1b) at 4,245 km².



a.

b.

Picture 1. City Pancevo in Serbia (a) and Pancevo in South Banat District (b).

Agricultural land accounts for 80.9%. Pancevo located 18 kilometers northeast of Belgrade. Pancevo is characterized by a temperate-continental climate, with an average perennial temperature of 11.3°C and precipitation 643 mm. Favorable conditions in the vegetation period have influenced the fact that a large part of the population is engaged in agriculture.

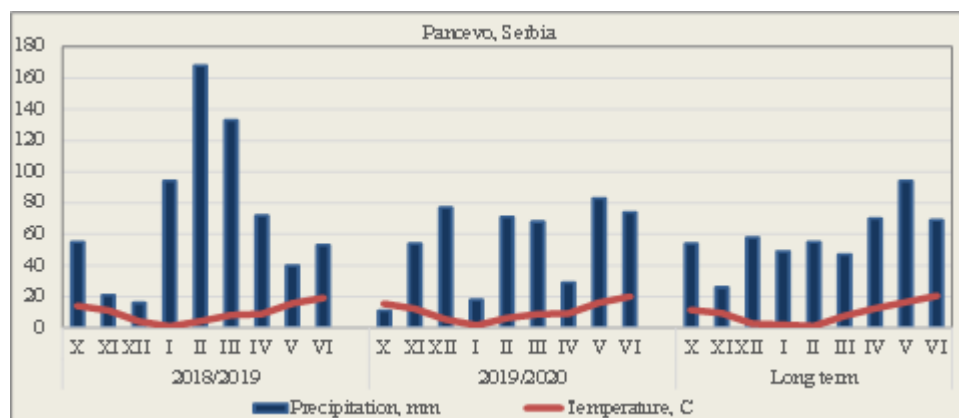


Figure 1. Precipitation and temperatures for the vegetation period of winter wheat and rye, Pancevo, Serbia

The amount of precipitation by months for the vegetation period of winter wheat in the years of testing, as well as the multi-year average (2010–2020) are shown in Graph 1. Mean multi-year temperatures for the vegetation period were 10.07 °C while the total vegetation precipitation was 522 mm. In the examined period, the average vegetation temperatures varied from 9.64 °C (2018/2019) to 10.5 °C (2019/2020) while the total vegetation precipitation varied from 485 mm (2019/2020) to 652 mm (2018/2019), (Figure 1).

Climate has a great influence on the growth and development of plants, so the elements of climate (light, heat, air and water) very often appear as a limiting factor (Popovic et al., 2012; 2020b; 2020c; Ljubicic et al., 2021). Significantly higher total precipitation for the vegetation period of winter cereals was in the first year of testing of 652 mm was higher compared to the precipitation for the multi-year average for the area of Pancevo by 130 mm while temperatures were lower by 0.33°C.

Yield of wheat and rye biomass

Based on the analysis of variance, it can be concluded that there are highly significant differences in biomass yield in regard to the genotype ($F_{\text{exp}}=937.75^{**}$) and years ($F_{\text{exp}}=28.07^{**}$) of investigation, Table 1.

The interaction of the investigated factors (Y x G) exhibits was significant affect in all cereals biomass yield ($F_{\text{exp}}=9.88^*$), ($p>0.05$). (Table 1). The present results confirm the opinion of many authors that the traits analysed are genetically determined but are strongly modified by the environment and weather conditions (Popovic et al., 2020c; Rajcic et al., 2020a; 2020b).

Wheat had a statistically significantly higher average biomass yield compared to rye, $p \geq 0.05$. The average biomass yield of the Ilina wheat variety was 2.79 t ha^{-1} , while the rye yield was 2.22 t ha^{-1} . Statistically significantly higher yield was achieved in the second year of the study 2019/2020 (2.56 t ha^{-1}) compared to the first year, 2018/2019 (2.46 t ha^{-1}), Table 2, Figure 2.

Table 1. The analysis of variance for the cereals biomass yield

Effect	Df	SS	MS	F	p-level
Intercept	1	75.350	75.350	72919.70**	0.000
Genotype	1	0.969	0.969	937.75**	0.000
Year	1	0.029	0.029	28.07**	0.000
Year x Genotype (YxG)	1	0.010	0.010	9.88*	0.014
Error	8	0.008	0.001		

*significant at 0.05; ** significant at 0.01;

Table 2. Descriptive Statistics of wheat and rye biomass yield, 2018/19-2019/20

Parameters	Level of Factor		No	Biomass yield Mean	Biomass yield Std.Dev.	Biomass yield Std.Err	Biomass yield -95,00%	Biomass yield +95,00%
Total			12	2,5058	0,3039	0,0877	2,3126	2,6989
Genotype;G	Wheat		6	2,7900	0,0357	0,0146	2,7524	2,8275
Genotype, G	Ray		6	2,2216	0,0906	0,0370	2,1265	2,3167
Year, Y	2018-2019		6	2,4566	0,3455	0,1410	2,0940	2,8192
Year, Y	2019-2020		6	2,5550	0,2794	0,1140	2,2617	2,8482
G x Y	Wheat	2018-2019	3	2,7700	0,0435	0,0251	2,6617	2,8782
G x Y	Wheat	2019-2020	3	2,8100	0,0100	0,0057	2,7851	2,8348
G x Y	Ray	2018-2019	3	2,1433	0,0450	0,0260	2,0313	2,2553
G x Y	Ray	2019-2020	3	2,3000	0,0100	0,0057	2,2751	2,3248

LSD	Biomass yield		
	G	Y	G x Y
0.05	0.043	0.043	0.061
0.01	0.062	0.062	0.088

The interaction of the examined factors had a statistically significant influence on the yield of wheat and rye biomass. Average rye biomass yields were 2.14 t ha^{-1} (2018/2019) and 2.30 t ha^{-1} (2019/2020) and were significantly lower than wheat biomass yields in 2018/2019 (2.77 t ha^{-1}) and in 2019/2020 (2.81 t ha^{-1}), Table 2, Figure 2.

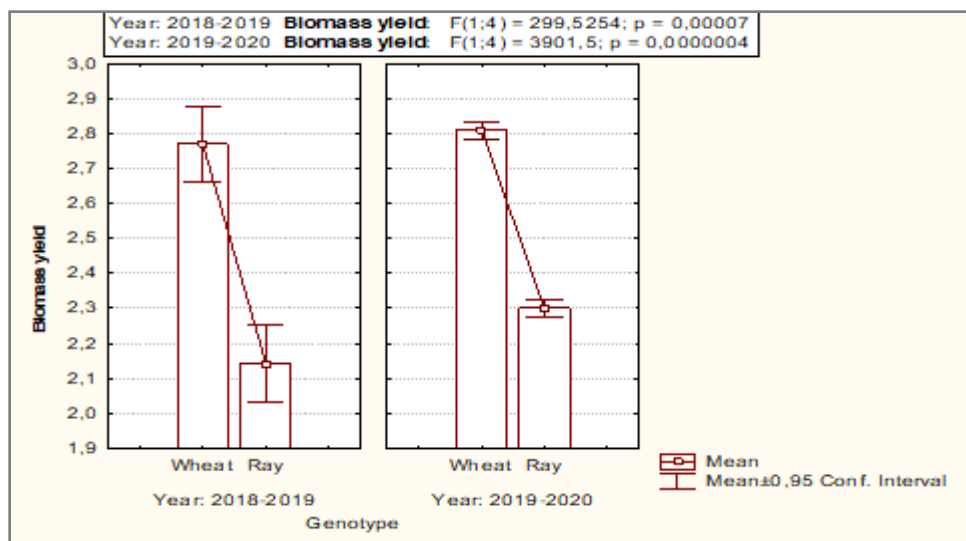


Figure 2. Wheat and rye biomass yield, Pancevo, 2018/2019-2019/2020

Biogas yield of wheat and ray

Based on the analysis of variance, it can be concluded that there are highly significant differences in biogas yield in regard to the genotype ($F_{\text{exp}}=3902.25^{**}$) and investigated years ($F_{\text{exp}}=5.32^*$). The interaction of the investigated factors (Y x G) exhibits was no significant affect in yield ($p>0.05$), Table 3.

Table 3. The analysis of variance for the wheat and ray biogas yield

Effect	Df	SS	MS	F	p-level
Intercept	1	102260	102260	56125.91**	0.000
Genotype, G	1	71098	71098	3902.25**	0.000
Year, Y	1	97	97	5.32*	0.005
Y x G	1	79	79	4.35 ^{ns}	0.070
Error	8	146	18		

^{ns}non significant; * significant at 0.05; ** significant at 0.01;

The present results confirm the opinion of many authors that the traits analysed are genetically determined but are strongly modified by the environment and weather conditions (Popovic et al., 2020c; Rakascan et al., 2021).

Wheat had a statistically significantly higher average biogas yield compared to rye, $p \leq 0.05$. The average biogas yield of the Ilina wheat variety was $368.89 \text{ m}^3 \text{ t}^{-1}$ while the rye yield was $214.94 \text{ m}^3 \text{ t}^{-1}$. Higher biogas yield was achieved in the second year of the 2019/2020 ($294.76 \text{ m}^3 \text{ t}^{-1}$) compared to the first year, 2018/2019 ($289.08 \text{ m}^3 \text{ t}^{-1}$) but the difference was not significant. The interaction of the examined factors did not have a statistically significant effect on the yield of wheat and rye biogas, Table 4, Figure 3.

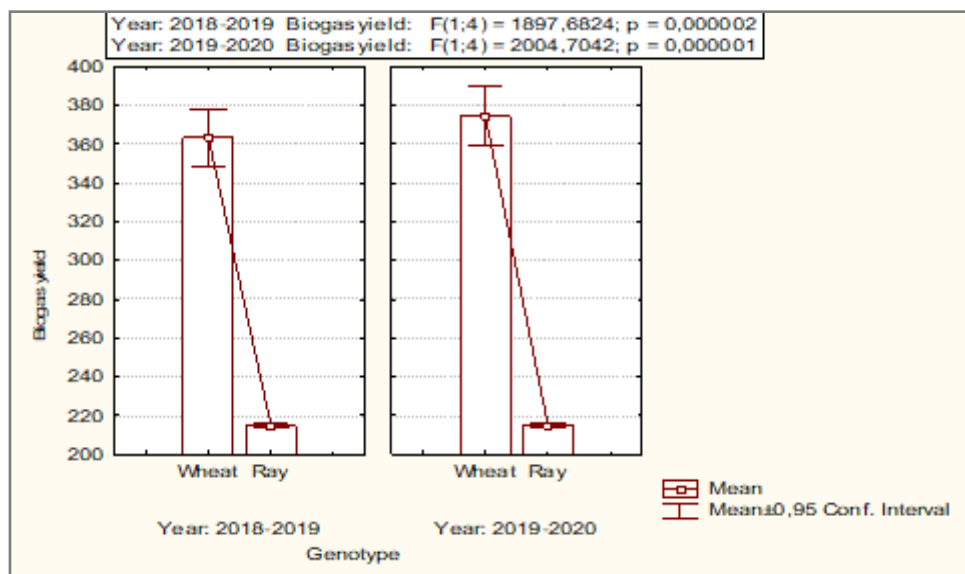


Figure 3. Wheat and rye biogas yield, Pancevo, 2018/2019-2019/2020

Table 4. Descriptive Statistics of wheat and rye biogas yield

Parameters	Level of Factor		No	Biogas yield Mean	Biogas yield Std.De v.	Biogas yield Std.Err	Biogas yield -95,00%	Biogas yield +95,00%
Total			12	291,919	80,577	23,260	240,723	343,116
Genotype, G	Wheat		6	368,892	8,013	3,271	360,483	377,301
Genotype, G	Ray		6	214,946	0,438	0,179	214,486	215,407
Year, Y	2018-2019		6	289,078	81,588	33,308	203,456	374,700
Year, Y	2019-2020		6	294,761	87,223	35,608	203,225	386,296
G x Y	Wheat	2018-2019	3	363,480	5,906	3,410	348,806	378,153
G x Y	Wheat	2019-2020	3	374,305	6,142	3,546	359,047	389,563
G x Y	Ray	2018-2019	3	214,676	0,339	0,195	213,833	215,519
G x Y	Ray	2019-2020	3	215,216	0,383	0,222	214,262	216,170

SD	L		
	Biogas yield		
	G	Y	G x Y
0.05	5.681	5.681	8.037
0.01	8.267	8.267	11.693

Correlations between tested parameters

The relationship between biomass yield and biogas properties in rye and wheat was presented using correlation analysis, using the Pearson linear correlation coefficient. A strong positive correlation between biomass and biogas yields was calculated ($r = 0.98^{**}$), table 5.

Table 5. Correlations between tested parameters

Parameter	Biomass yield	Biogas yield	Temperature	Precipitation
Biomass yield	1.00	0.98 ^{**}	0.17 ^{ns}	0.17 ^{ns}
Biogas yield	0.98 ^{**}	1.00	0.04 ^{ns}	0.04 ^{ns}

^{ns} non significant; ^{**} significant at 0.01.

The use of correlation coefficient analysis in the wheat and rye, simplify dependable classification of bread wheat and rye, the identification of the superior genotypes and their relationship with bio morphological traits in future breeding programs.

Wheat genotypes reacted differently in the two growing seasons, giving high results in most of the traits that contribute directly to grain yield during crop years (Ristić et al., 2009; Popovic et al. 2020c; Mandić et al., 2020; Đurić et al., 2020; Miroslavljević et al., 2018; 2019). Similar results were obtained in the research of Milanovic et al. (2019; 2020); Popovic et al., (2020c) and Rakascan et al. (2019a; 2019b; 2021).

The present results confirm the statement of many authors that the traits analyzed, and their correlations are genetically determined but are strongly modified by the nutrient status of the environment and weather conditions (Djekic et al., 2014; Lakic et al., 2018; Rajicic et al., 2020a, 2020b).

Grain yield is one of the most important and complex traits in plant breeding. Knowledge about crop development, growth and dry matter accumulation enables the appropriate selection of genotypes and field technology. Growing season, sowing date, variety and sowing date × variety interaction had significant influence on crop biomass at anthesis and grain yield of wheat and barley. Dry matter translocation was in positive correlation with dry matter content at anthesis. Dry matter accumulation across growing seasons had a typical sigmoid pattern in every combination of sowing date and variety. These results indicated that during the selection of small grain cereals, it is necessary to develop genotypes characterized by higher leaf number and increased biomass, i.e., genotypes that in optimal sowing dates, have an adjusted phenological development to the agro-ecological conditions of the Panonian plane (Miroslavljević, 2015). The variety itself cannot give a high yield without the application of all agrotechnical measures, to which we strive for primarily due to the high wheat importance. Wheat yield depended more on sowing time, and the influence of sowing time, variety, and interaction of these two factors on yield were statistically significant. The highest yield was registered (8.87 t ha⁻¹), while the average yield achieved by this micro experiment was 8.46 t ha⁻¹ (Veliborović

et al., 2020). Župunski et al. (2021) results indicate the specificity of the years, the locations, and the responses of the varieties to mycobiota associated with winter wheat grain. Swedish scientist MacKey (1966) pointed out that the yield is very relative term, and it is always determined by variety, ecological conditions and level of the sole production. Borojevic and Cupina (1969), by testing yield components in the case of different wheat genotypes, concluded that the greater number of various traits have more decisive role in yield making in the case of various genotypes. It is the result of interaction between traits within one genotype as well as between genotype and environmental factor.

CONCLUSIONS

The growth trend of alternative fuels has been evident in the world in recent years, thanks to the development of new technologies for processing bio-waste into energy sources.

Wheat had a statistically significantly higher average yield of biomass and biogas compared to rye. Based on the analysis of variance, it can be concluded that there are highly significant differences in biomass yield in regard to the genotype ($F_{\text{exp}}=937.75^{**}$) and years ($F_{\text{exp}}=28.07^{**}$) of investigation.

The average biomass yield of the Ilina wheat variety was 2.79 t ha^{-1} , while the rye yield was 2.22 t ha^{-1} . Statistically significantly higher yield was achieved in the second year of the study 2019/2020 (2.56 t ha^{-1}) compared to the first year, 2018/2019 (2.46 t ha^{-1}).

There was a strong positive correlation between biomass yield and biogas yield.

Circular economy is an approach that integrates the economy and the waste management system and is a great chance for the development of each country.

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COMPARING PIXEL- AND OBJECT- BASED FOREST CANOPY GAPS CLASSIFICATION USING LOW-COST UNMANNED AERIAL VEHICLE IMAGERY

SUMMARY

Forest canopy gaps are an important indicator of ecosystem dynamics. Gap sizes can vary because of several agents, and the spatial distribution is related to abiotic factors. The interest in the study of this forest attribute is old, but the difficulties to detect these areas in situ and with the use of satellite imagery hinder this research approach. Thus, we explore the use of high spatial resolution images obtained with RGB boarded in a multirotor unmanned aerial vehicle (UAV) to evaluate the best method to mapping the forest canopy gaps in Brazil. For this, were utilized the pixel- and object-based approaches, and the algorithms Random Forest (RF) and Support Vector Machine (SVM). The results showed that the ortophotomosaics can overcome the disadvantages of study the forest canopy gaps from conventional methods and reduce the complexity and costs to obtain reliable data of forests remnants. The RF and the pixel-based classification were the best combinations, with an overall accuracy (OA) of 93% in the period of study. However, the SVM presented a satisfactory accuracy to classify the forest canopy gaps, with the precision of user (PU) ranging from 86% to 98% and measure F from 85% to 96%. Therefore, was confirmed the potential of low-cost UAVs boarded with RGB sensors in this research proposal, and the results are promising for future studies.

Key words: Structure from Motion, Random Forest, Support Vector Machine, Forest Remnant, Conservation, Brazil

INTRODUCTION

Gaps in forests canopies represent the result of ecological disturbances and are a key element for understanding forest structure and dynamics (Karki and Hallgren, 2015; Mohammadi *et al.*, 2021). The formation of these openings in the forests varies in size because of several agents, such as the wind, diseases, fire,

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and cyclones (Bazzaz, 1983). Nevertheless, the spatial distribution of central abiotic factors also plays an important role in the regulation of the formation of the gaps (Getzin *et al.*, 2014).

Therefore, the detection of these areas is an important alternative to monitor the forests remnants because the gaps influence the composition and species richness from microhabitats. Thus, it affects the quality and quantity of available resources, such as light (Kuuluvainen and Linkosalo, 1998), which promotes the natural regeneration of trees and the diversity of understory biota (Burton *et al.*, 2014).

The interest of ecologists in understanding these processes and recognizing the dynamics involved is old (Fisch and Ponzoni, 1995), but the spatial resolution of the satellite's images and the difficulty of displacement on the ground due to the disturbance of these areas, restrict the studies.

In this scenario, unmanned aerial vehicles (UAVs) have become an affordable alternative, capable of providing the flexibility and resolution necessary to accurately map the forests (Fassnacht *et al.*, 2003; Chianucci *et al.*, 2015). The combination of these platforms with computer vision algorithms ensures the generation of high-quality products, such as orthophotomosaics and three-dimensional (3D) models, which have the potential to detect and identify flora classes (Felix *et al.*, 2021). Nonetheless, the UAVs can be equipped with different sensors, capable of acquiring information from different portions of the electromagnetic spectrum, like the visible bands (RGB), red edge, and near-infrared (NIR) (Grybas and Congalton, 2021).

According to Castillo *et al.* (2012), the difference in the use of UAVs is the increase in accessibility, performance, and precision in the acquisition of data and orthoimages in high resolutions. Because of this methodological configuration, the limitations of traditional remote sensing techniques are overcome, mainly about significant errors in volumetric calculations.

Studies such as that of Wallace *et al.* (2016), Prošek and Šímová, (2019), and Olivetti *et al.* (2020), have already recognized this potential and achieved satisfactory results in the use of UAVs in different environmental contexts. However, precision agriculture is still considered the area with the greatest potential for its application (Jorge *et al.*, 2014).

Thus, in this study we aimed to demonstrate how images acquired with RGB sensor carried on a low-cost UAV can be used for forests gap studies; two different classification approaches (pixel- and object-based) and the algorithms Random Forest (RF) and Support Vector Machine (SVM) were utilized.

MATERIAL AND METHODS

The study area is in the municipality of Lavras, state of Minas Gerais (Figure 1). Forest remnant has 2.81 ha and represents a typical Cerrado (Savanna) physiognomy, with 19 families, 38 species, and 38 genera ($H' = 3.28$), with the exclusive occurrence of individuals such as *Bowdichia virgiloides*, *Dalbergia miscolodium*, and *Qualea grandiflora* (Pereira *et al.*, 2010).



Figure 1. Location map of the study area in the municipality of Lavras, Brazil.

According to the Köppen classification system, the climate of the study area is mesothermal tropical (Cwb) (Sparovek *et al.*, 2007). The average elevation is 918 m, and the mean annual precipitation is 1529.7 mm, with soil classified as dystroferic Red Latosols (FEAM, 2010).

The region is part of the upstream portion of the Rio Grande watershed and makes up the geomorphological Atlantic Plateau unit in Varginha Complex crystalline rocks (CPRM 2014). The vegetation is characterized by the transition of the Atlantic Forest to the Cerrado (Savanna), with the presence of the remnants of Montana Semi Deciduous Forest (Oliveira-Filho *et al.*, 2001).

PROCEDURE

1. Unmanned aerial vehicle (UAV):

We used the multicopter Phantom 3 (Professional) with an RGB sensor, camera model Sony EXMOR $\frac{1}{2.3}$ ", which captures images in real colour with lens 94° FOV 20 mm (Figure 2).

Considering that the occurrence of gaps is seasonal, being more frequent during the rainy season when the fall of trees and branches is also amplified by the strong winds (Sarukhán, 1978), two flights were scheduled. One to run in the middle of the dry season (24 August 2017) and the other after the rainy season (1 February 2018), at a height of 60 m and with 80% forward and side overlap. The grid adopted was 50 x 50 m and the mapping was planned and executed using the GCS (Ground Control Station) Pix4DCapture.



Figure 2. DJI Phantom 3 equipped with an RGB sensor.

The images were processed using the commercially available structure-from-motion (SfM) Agisoft Photoscan Professional® v1.2.7 software with the configurations: “*Align Photos*” = high; “*Accuracy*” = generic / pair pre-selection and 40 000 points features per image; “*Tie points*” = 10 000. No ground control points (GCPs) were used for image orthorectification.

2. Segmentation:

Segmentation considers the radiometric information of the pixels, the semantic properties of each segment, and other background information that describes the connection of adjacent pixels, such as the intensity, texture, shape, and dimensional relations (De Luca *et al.*, 2019).

Thus, the object-based image analysis (OBIA) aimed to group pixels into homogeneous classes, allowing the use of multiple descriptive statistics and contextual information during the classification process (Blaschke, 2010).

In this study, the OBIA method was based in the multi-resolution segmentation algorithm implemented on eCognition 8.3. After iterative tests, the parameters selected were: “*scale*” = 40; “*shape*” = 0.3 and “*compactness*” = 0.8.

Since the texture features are one of the important characteristics used for identifying objects (Haralick, 1979), the measure GLCM was implemented. Thus, after the segmentation, the mean, the standard deviation, the homogeneity, the entropy of the Red band, the maximum difference, and the brightness were extracted for each sample, resulting in 12 attributes. Therefore, 200 objects were selected as training data, which were grouped into 5 classes: (1) bare land, (2) branches, (3) canopies, (4) gaps, and (5) shadows.

3. Pixel-based approach:

Reference polygons were selected by visual interpretation of the orthophotomosaics, using the true colour composition. A total of 200 reference polygons were selected as training samples to five classes described in section 2. Nonetheless, the variables extracted for each sample were only the mean of the bands, resulting in three attributes.

4. Orthophotomosaics classification:

The RF algorithm (Breiman, 2001) is a non-parametric and robust algorithm used for images classification. A couple of studies presented satisfactory results on the employment of RF in land use and species classification approaches (Belgiu and Dragut, 2016).

In this study, the RF was grown with the parameters “*n_{tree}*” = 500 and “*m_{try}*” with the square root of the number of the variables included in the model as described in sections 2 and 3.

On the other hand, the SVM (Cortes and Vapnik, 1995) are based on statistical learning theory, defining the optimal hyperplane as a linear decision function between the vectors of two classes (Deur *et al.*, 2020). We used the radial basis function kernel in this study.

Thus, each singular date of imagery was classified in R open-source statistical programming environment, and the training samples were randomly divided into training (70%) and validation (30%) datasets.

The accuracy of these classifications was assessed using the error matrix approach. After, were obtained the overall accuracy (OA), the accuracy of classes, and the kappa index (Congalton, 1991). For each matrix, the precision of the classes was determined by calculating the precision of user (PU) and producer (PP) (Story and Congalton, 1986), and to summarize PU and PP in a single metric, measure F was calculated (Equation 1).

$$F = 2 * \frac{(PU*PP)}{(PU+PP)} \quad (\text{Equation 1})$$

RESULTS AND DISCUSSION

UAV imagery reached 2 cm spatial resolution, which facilitates the detection of classes by visual interpretation using the true colour composition.

The highest pixel-based classification accuracy was obtained for the RF algorithm on both dates, with OA = 93%, and kappa = 0.88 and 0.90, respectively (Table 1 and Table 2). Concerning the forest gaps, the highest accuracy was obtained for RF (PU ranged from 92% to 94% and the measure F ranging from 93% to 95%).

Although the SVM presented the worst result than RF in the classification accuracy for forest gaps (PU ranged from 92% to 98% and the measure F ranging from 90% to 96%), the performance of the algorithm was satisfactory.

Table 1. Accuracy assessment of pixel-based classification with RF and SVM on 24 August 2017.

RF								
Prediction	B.L.	Branches	Canopies	Gaps	Shad.	Total	PU	F
Bare land	975	28	3	15	46	1 067	91%	85%
Branches	75	10 298	664	53	7	11 097	93%	92%
Canopies	13	908	25 331	161	29	26 442	96%	96%
Gaps	65	91	171	39 540	3 089	42 956	92%	95%
Shadows	83	63	3	532	1 638	2 319	70%	46%
Total	1 211	11 388	26 172	40 301	4 809	OA = 93% Kappa = 0.88		
PP	80%	90%	97%	98%	34%			
SVM								
Bare land	4 014	453	1 768	340	0	6 575	96%	93%
Branches	35	8 559	6 642	610	0	15 846	86%	47%
Canopies	42	15 924	21 952	413	0	38 331	67%	69%
Gaps	294	926	251	18 403	0	19 874	92%	90%
Shadows	0	0	0	900	2 345	3 245	72%	84%
Total	4 385	25 862	30 613	20 576	2 345	OA = 35% Kappa = 0.50		
PP	91%	33%	71%	89%	100%			

PP: precision of producer; PU: precision of user; and F: measure F.

Table 2. Accuracy assessment of pixel-based classification with RF and SVM on 1 February 2018.

RF							
Prediction	Bare land	Branches	Canopies	Gaps	Total	PU	F
Bare land	3 967	60	29	79	4 135	96%	93%
Branches	110	23 821	1 590	417	25 938	92%	92%
Canopies	59	1 491	28 649	556	30 755	93%	93%
Gaps	249	490	345	18 714	19 798	94%	94%
Total	4 385	25 862	30 613	19 766	OA = 93% Kappa = 0.90		
PP	90%	92%	93%	94%			
SVM							
Bare land	951	9 658	6 370	391	17 370	5%	9%
Branches	54	20 661	4 653	233	25 601	80%	72%
Canopies	11	942	24 940	4	35 897	69%	60%
Gaps	18	33	142	11 565	11 758	98%	96%
Total	1 034	31 294	46 105	12 193	OA = 31% Kappa = 0.17		
PP	92%	66%	54%	95%			

PP: precision of producer; PU: precision of user; and F: measure F.

In the case of the OBIA classification, the RF also showed the best results in both dates and in the detection of the forest gaps (Table 3 and 4). The OA ranging from 78% to 88%, the PU from 85% to 89%, with a measure F of 96% and 90%.

Table 3. Accuracy assessment of object-based classification with RF and SVM on 24 August 2017.

RF								
Prediction	B.L.	Branches	Canopies	Gaps	Shadows	Total	PU	F
Bare land	0	0	0	0	0	0	100%	0%
Branches	1	12	3	0	0	16	75%	79%
Canopies	1	5	17	0	0	23	74%	85%
Gaps	0	0	0	18	3	21	85%	96%
Shadows	0	0	0	0	0	0	100%	0%
Total	2	17	20	18	3	OA = 78% Kappa = 0.68		
PP	0%	70%	85%	100%	0%			
SVM								
Bare land	0	0	0	0	0	0	100%	0%
Branches	0	8	2	0	0	10	80%	87%
Canopies	2	9	18	0	0	29	62%	66%
Gaps	0	0	0	18	3	21	86%	89%
Shadows	0	0	0	0	0	0	100%	0%
Total	2	17	20	18	3	OA = 73% Kappa = 0.61		
PP	100%	95%	72%	93%	100%			

PP: precision of producer; PU: precision of user; and F: measure F.

Table 4. Accuracy assessment of object-based classification with RF and SVM on 1 February 2018.

RF							
Prediction	Bare l.	Branches	Canopies	Gaps	Total	PU	F
Bare land	2	0	0	0	2	100%	66%
Branches	0	16	0	0	16	100%	94%
Canopies	1	1	20	2	24	89%	92%
Gaps	1	1	1	15	18	93%	90%
Total	4	18	21	17	OA = 88% Kappa = 0.83		
PP	50%	89%	95%	88%			
SVM							
Bare land	1	0	0	0	1	100%	40%
Branches	0	6	3	0	9	93%	48%
Canopies	2	12	18	4	36	54%	66%
Gaps	1	0	0	13	14	97%	85%
Total	4	18	21	60	OA = 63% Kappa = 0.46		
PP	25%	33%	86%	76%			

PP: precision of producer; PU: precision of user; and F: measure F.

These results indicating that the best approach of the study was the pixel-based classification with the RF, and the RGB sensor is a feasible alternative to monitor the forest dynamics (Figure 3).

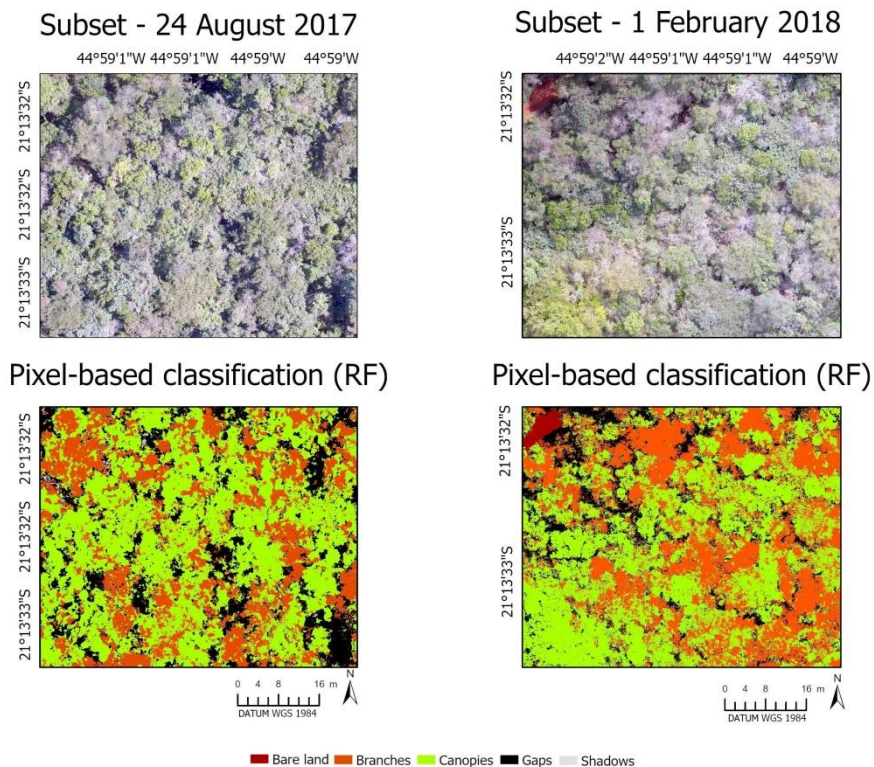


Figure 3. Example subsets of pixel-based classification with the Random Forest algorithm in both dates of study.

According to Deur *et al.* (2020), the OBIA was the best method to work with high spatial products, but as demonstrated in our results the performance was lower than the pixel-based method. A couple of studies also indicated that the SVM algorithm is the best alternative to OBIA classification (Belgiu and Dragut, 2016), which wasn't confirmed in this research because the SVM showed the worst results obtained with the two classification methods.

Despite that, our results confirmed the potential reported to other studies in the evaluation of forest canopy gaps with UAVs (Bagaran *et al.*, 2018, Bourgoïn *et al.*, 2020), and reinforce the capacities of low-cost equipment configuration in these interest front.

The spatial resolution of orthophotomosaics allowed the identification of a significant number of small gaps ($\leq 1\text{ m}^2$), especially in February 2018. In the last years, the discussion on the minimal gap size restricts the threshold from 1 m^2 to 5 m^2 (Nieschulze *et al.*, 2012, Boyd *et al.*, 2013). Therefore, our results point to the importance of a better evaluation about the metrics adopted in the studies of forest canopy gaps from remote sensing. However, we agree with Tanaka and Nakashizuka (1997) and Getzin *et al.* (2014), that the monitoring of small gaps

should be analyzed in the long-term, because the disturbances around these areas cannot be explained simply as gap expansion but should be considered the regeneration and successional dynamics of the understory.

Thus, we recognize the promising results obtained with the use of UAVs, still, we emphasize that the use of these technologies does not replace the evaluation of these areas *in situ*. In the case of Brazil, with the recent availability of pan sharpened very high spatial resolution of CBERS-4A satellite (2 m), as well as the free Planet satellite imagery (3-5 m), the integrated use of these platforms can allow the monitoring of long-term forest dynamics. Furthermore, this configuration offers a great opportunity to overcome the satellite's limitations in other areas and can be an alternative to evaluate the forest canopy gaps with the climate changes.

Last, this research compared the performance of two methods to classification and identifying forest attributes, but we do not disqualify the use of the SVM or the OBIA method for future studies. After all, the algorithm and classification method are historically consolidated and can contribute to several mapping approaches. Because, as confirmed in Table 1 and Table 2, the SVM presented a highest accuracy for gaps classification with the pixel-based method.

Thus, our results represent directions for future researchers aiming the mapping and monitoring forests attributes, especially in Tropical areas.

CONCLUSIONS

The use of unmanned aerial vehicles to detect and monitoring forest dynamics can reduce the costs and provide promising results in mapping the forest canopy gaps. RGB sensor represented a feasible alternative to overcome the limitations of satellite data, but the combined use of these platforms provides a great opportunity to maintain the landscape. The Random Forest algorithm confirmed its robustness and capabilities to use in different contexts. Nonetheless, Support Vector Machine also represents an alternative for future research aiming at the study of forest canopy gaps.

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BIOACCUMULATION OF 18 TRACE METALS IN MUSCLE AND EXOSKELETON IN THE NOBLE CRAYFISH (*ASTACUS ASTACUS* L.) IN THE RIVER ZETA (MONTENEGRO)

SUMMARY

This study assessed the level of heavy metals accumulation in abdominal muscle and exoskeleton on of *Astacus astacus* L. collected from Zeta River in July, with the aim of predicting health risk effect on human consumers. Noble crayfish samples were collected from four different sites along the river. The concentration of metals in the muscle and chitin of the Noble crayfish was determined using atomic absorption spectrophotometer. The exoskeleton showed a decidedly higher content of most analysed trace elements with respect to muscles (two- to fourfold higher for Al, Ca; two-to eightfold for Fe and ten times higher for Sn and Mn). Hg, K, Mg and Zn were found in higher concentrations in the abdominal muscle in Noble crayfish from the River Zeta. Nonsignificant differences were found between muscles and exoskeleton for As, Cu, K, Na, Ni, and Si (except first locality). Muscle content analysis had indicated that sampling point I (first) had the lowest concentration and sampling point IV (the last) had the highest concentration of accumulated heavy metals. It was believed that domestic activities around the river the major contributing factor to the accumulation of toxic heavy metals in muscle and carapace sample analysed, it is recommended that intervention relevant authorities are needed to curtail chemical degradation of the aquatic biota over a period of time. The most of trace element concentrations found in crayfish tissues were in the range considered harmful to human health (except for Na, Ni, Sn and Cr). We hypothesize that *A. astacus* in the Zeta River aquatic communities can act as a vector of pollutants, as crayfish can transfer their relatively high amount of heavy metals to higher trophic levels.

Keywords: Bioaccumulation, heavy metals, crayfish muscle Exoskeletonon, pollution, Montenegro.

INTRODUCTION

The Noble crayfish (*Astacus astacus* L.) stands as an important food in many parts of the Europe, being a rich source of protein. It is a commercial

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species in Montenegro. In the past, it was hunted in larger quantities (Marić and Rajković, 2004), and lately in much smaller ones, but there is no reliable data on that. In general, commercial hunting of all freshwater species is in significant decline (Marić, 2018; Pešić *et al.* 2019), and the causes have not been studied in detail. The health safety of aquatic organisms used for commercial purposes has also not been well and comprehensively studied. Only some species of fish have been studied in more detail from the aspect of bioaccumulation of heavy metals in muscles (Rakočević *et al.* 2018). The Noble crayfish has not been studied from this aspect. The commercial aspect and the lack of data on the quality of meat of this type are the main reasons for the research of metals presented in this paper.

In the least hundred years, a significant increase in the concentration of metals in the environment has been recorded (Vamerali *et al.* 2010). Heavy metals of natural and anthropogenic sources are released into aquatic ecosystems, where they pose a serious threat because of their toxicity, long persistence, bioaccumulation and biomagnifications in the food chain (Zhou *et al.* 2008). Aquatic invertebrates are sensitive to metals in their environment and are mostly used to assess aquatic ecosystem quality (Bonada *et al.* 2006). They can accumulate metals directly from the sediments or by food and, based on their life cycle, some species can accumulate more elements than the others (Pourang 1996). Heavy metals affect living organisms in various ways; some have essential functions (e.g. Cu, Mn and Zn) contributing to the maintenance of a good health in humans and animals (Rainbow 2007) and are toxic only at high levels, whereas others are xenobiotic: non-essential metals as Pb and Hg do not play any role in metabolism and are toxic even at low concentrations (Kouba *et al.* 2010). The aim of present work was to characterize heavy metal content in selected tissues of noble crayfish samples collected from different sites of the River Zeta in order to assess bioaccumulation mechanisms, evaluate whether these concentrations could be considered harmful for human health and, finally, to identify possible sources of contamination in the study area. We exploited the selective metal bioaccumulation in two different tissues of *A. astacus*, exoskeleton, and abdominal muscle, which is the edible part. There is very scarce information on heavy metal distribution in biota of this area, in spite of the presence of many industrial and agricultural activities.

MATERIAL AND METHODS

Area and habitat study

Nikšić field is situated in the western part of the Republic of Montenegro. It is situated between 18° 30' to 19° 10' of eastern geographical longitude and 42° 42' to 42° 53' of northern geographical latitude on the altitude of around 600-630m (Figure 1). The main water course of Nikšićko polje (field) is the so called Gornja Zeta (the Upper Zeta). The length of the River Gornja Zeta is around 15 km counting from the confluence of the Rastovac and the Sušica, than it disappears under the ground and appears again on the spring Glava Zete on peak

elevation of 50m. There are three artificial lakes Krupac, Slano and Liverovići. The Noble crayfish also live in them.

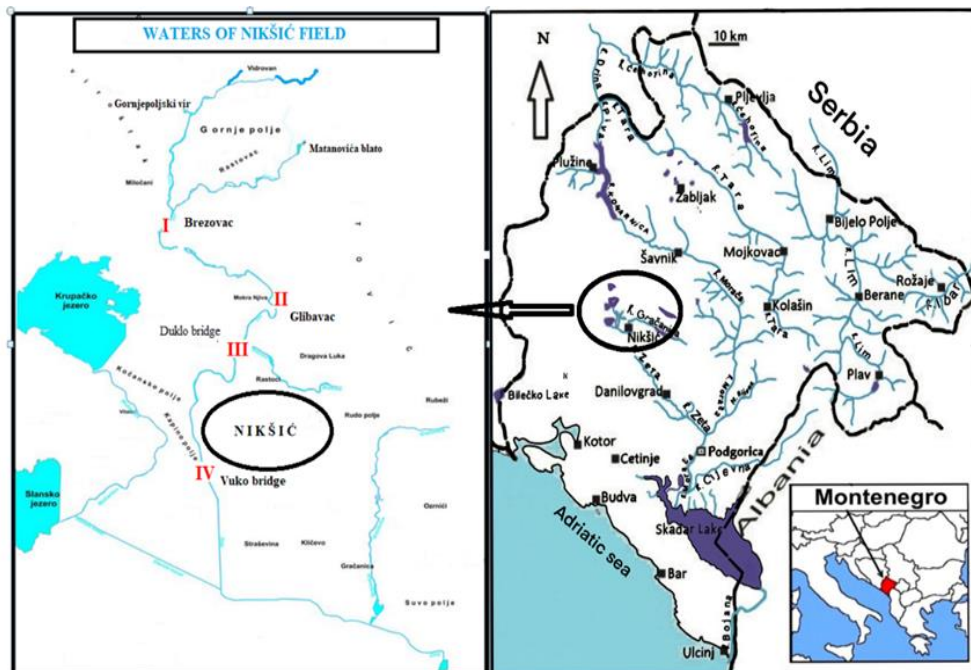


Figure 1. Map of water bodies in Nikšić field (sampling sites marked with Roman numerals)

The width of the riverbed is 7-25 m, and the water depth is 0.2 to 5 m. Different trees and shrubs grow on the banks of the river, mostly willows (*Salix* spp.) and poplars (*Populus* spp.). The bank of the river is in some places overgrown with reeds - *Phragmites* sp. The riverbed has different water depths, the lotic and lentic areas alternate. The bottom of the river is covered with aquatic vegetation (70%) which consists mainly of species of the genus *Potamogeton*, to a lesser extent the species *Miriofilum* sp. and *Ranunculus* sp. The fauna of the bottom of the river Zeta consists of representatives of Gastropods, Oligochaeta and insect larvae: Ephemeroptera, Plecoptera, Trichoptera, Chironomidae, Simuliidae, etc. (Marić and Rajković, 2004). According to Marić (2019) were found six species of fishes: *Thimallus thimallus*; *Salmo farioides*; *Salmo labrax*; *Oncorhynchus mykiss* *Squalius plaryceps*; *Phoxinus* sp.

Noble crayfish were collected from four localities on the Zeta River (Figure 1) the first locality, Gornje Polje, is located in the immediate vicinity where two rivers, Rastovac and Bistrica, join and form the river Zeta. There are no industrial pollutants in this area. The 2nd locality (II), Mokra Njiva, 2-3 km away from the main road Nikšić - Plužine. The Brezovik hospital located upstream 2 km from this site, and it has a collector for water purification. The

locality Duklov most (III), is located in the city zone. Upstream from this locality, the river Bistrica flows into the river Zeta. It flows through the industrial zone of the city and brings waste of different origin. The fourth locality (IV), Vukov most (bridge), is just below the city. All industrial waters and household waters upstream reach in the river.

Crayfish collection and measurements and statistical analysis

Our study was conducted in the summer (July) 2013 using a commercial fishing gears – the traps or fyke nets in the area of the upper course of the Zeta River, Montenegro. Researching area included 4 localities between which the distance is about 3 km. A total of 99 specimens of the Noble Crayfish (*Astacus astacus* L.) in the River Zeta were caught. All specimens (fresh) were measured for total length (TL, in cm) and weight (W, total wet weight in g) to the nearest 0.01g by an electronic balance. The total length (TL) of each specimens (measured using digital caliper to the nearest 0.01 mm) was taken from the beginning of the rostrum to the end of the telson. All specimens (sexually mature) of the Noble Crayfish caught at four site were larger than 9.5 cm. Differences between 4 sites and samples were analysed by Analysis of Variance (ANOVA). The statistical analyses were considered at a significance level of 1% ($P < 0.01$).

Tissue samples of the caudal (tail) muscle and exoskeleton (from carapace) were taken, than milled using an electric grinder and stored in airtight polyethylene bottles at $-18\text{ }^{\circ}\text{C}$ until chemical analyses. All chemical analyses were performed in laboratory of Center for Eco-toxicological Research (CETI, Podgorica). Three parallel determinations were carried out for each parameter. Concentrations of analysed trace elements were expressed on a wet weight basis, as mg kg^{-1} . The limits of quantification (LOQ) were: 0.1 mg kg^{-1} for Pb; 0.01 mg kg^{-1} for Cd; 0.06 mg kg^{-1} for As; 0.025 mg kg^{-1} for Co and Cr; 0.02 mg kg^{-1} for Ni; 0.2 mg kg^{-1} for Fe, Mn, Cu and Zn; 0.001 mg kg^{-1} for Hg; and 5 mg kg^{-1} for Ca and Mg. Detailed procedure was described by Rakocevic et al (2018). Data analyses were performed with using Statistica 8.0 software (StatSoft, USA). Data are given as Mean \pm SD (standard deviation).

RESULTS

The Noble crayfish caught ranged in total length from 9.50 to 14.60 cm and in weight from 23.50 to 125.90 g. No significant differences were found between the average length and weight of the investigated Noble crayfish from four localities ($P > 0.05$).

The concentrations of the relevant metals (15 metals) analysed in the muscle and exoskeleton - carapace from four localities in the Zeta River are presented in Table 1. Cadmium, Pb and Mo, if present, were in concentrations below the detection limit. A wide range of concentrations of some metals were found in the analysed tissues in the muscle: As < 0.025 -16.0, Ca 25.2-116.1, Hg 33.1-97.1, Si 15.6-43.1 and in the exoskeleton Al 9.6-68.9, Ca 68.9-141.1, Fe 26.7-58.5, Mn 37.4-84.4.

A narrow range of concentrations was detected for each of the other trace metals in the muscle Al 3.7-13.1, Cr undetected-1.1, Cu 8.6-21.7, Fe 6.0-17.9, K 2.3-8.2, Mn 2.2-14.2, Mg 200.1-253.5, Na 0.8-2.2, Ni 0.04-0.14, Sn 0.2-1.6, Zn 17.6-19.8 and in the exoskeleton As 7.8-15.6, Cu 17.5-22.2, Hg 6.2-15.1, K 1.9-3.3, Mg 1.0-2.5, Na 1.9-2.7, Ni 0.03-0.06, Sn 27.9-40.8, Si 40.6-46.8, Zn 11.3-18.8. Cr was found only in the second locality in both carapace and abdominal muscle but was higher in the latter.

As shown in table 1, Al, Cu, Ca, Fe, Mn, Sn and Si concentrations were significantly higher in carapace of *A. astacus* than abdominal muscle. Mg, Hg, K and Zn mainly accumulated in crayfish muscle. Only two metals (Hg and Mg) were significantly higher in abdominal muscle of *A. astacus* than carapace. Mg primarily accumulated in crayfish abdominal muscle at similar levels for all sites (Tables 1). No significant differences were found for the other trace elements content among the sampled tissues.

Table 1. Concentrations of 15 metals in the muscle and chitin- exoskeleton samples from different locations. Data are given as Mean±SD.

Locality	1.		2.		3.		4	
	muscle	chitin	muscle	chitin	muscle	chitin	muscle	chitin
Al [#]	3.7±2.01	9.6±5.50	13.1±6.33	68.9±9.82	11.4±6.23	20.6±6.05	8.5±5.88	49.8±7.54
As	< 0.025	7.8±4.82	16.0±7.45	10.4±7.23	12.8±7.03	10.8±6.24	12.2±6.06	15.6±6.81
Ca [#]	25.2±5.87	68.9±12.34	36.7±8.79	141.1±15.38	37.3±7.54	56.2±10.16	116.1±12.89	49.9±10.84
Cr	/	/	1.1±0.32	<0,02	/	/	/	/
Cu [#]	8.6±2.88	17.5±3.61	12.9±3.98	22.2±5.04	21.7±6.32	17.9±3.76	15.4±4.13	20.7±4.87
Hg [#]	85.9±43.58	10.7±4.65	97.1±34.12	12.5±6.72	59.3±9.58	6.2±3.61	33.1±8.62	15.1±6.81
Fe [#]	6.0±5.21	58.5±41.86	13.2±10.29	48.8±40.34	14.4±12.65	26.7±20.43	17.9±16.82	38.4±28.21
K	2.3±1.11	2.1±0.93	4.0±1.74	3.3±1.23	4.0±1.65	2.0±0.90	8.2±4.66	1.9±0.96
Mn [#]	2.2±0.55	60.9±20.11	3.7±1.08	37.4±6.24	6.8±2.45	63.1±8.33	14.2±4.56	84.4±25.66
Mg [#]	200.1±43.76	1.6±0.22	222.8±38.93	2.5±0.72	242.0±44.37	1.6±0.75	253.5±56.88	1.0±0.20
Na	0.8±0,21	1.9±0.28	1.7±0.31	2.7±0.33	2.1±0.32	2.0±0.24	2.2±0.43	2.3±0.36
Ni	0.04±0.02	0,05±0.4	0.10±0.09	0.05±0.03	0.14±0.12	0.06±0.03	0.05±0.03	0.03±0.02
Sn [#]	0.2±0.09	27.9±18.65	0.31±0.08	38.6±25.82	0.4±0.09	39.7±22.41	1.6±0.02	40.8±19.94
Si [#]	15.6±10.17	46.8±12.03	43.1±20.65	40.6±10.56	24.4±10.28	43.0±11.62	34.9±12.88	40.9±14.53
Zn	18.0±9,78	13.3±5.49	19.8±8.76	15.7±6.38	17.6±7.34	11.3±5.11	18.9±10.56	18.8±7.68

The concentrations of fifteen elements in the noble crayfish were examined and the results showed the wide variations for the concentration of the trace of almost all elements among four localities (Table 1). The concentration of heavy metals in muscle showed significant differences between sampling sites (one-way ANOVA, all $p < 0.01$), and was higher at Vukovog mosta (4 sites), while first sites exhibited a lower metal pollution scenario. As shown in table 1, the smallest amount were found for twelve metals (80%): As; Al; Cu; Ca; Fe; Mn; K; Ni; Na; Sn; Si and Mg in the muscles, while the highest values were not found at the first locality. The concentrations of Fe and Si in exoskeleton of crayfish from first

locality was highest comparing to the other three localities, and at the fourth only for Hg and Mn.

The concentrations of four essential elements (Mn, Fe, Cu and Zn) in the noble crayfish showed a similar range of concentrations on the longitudinal profile. Observed on the longitudinal profile, Mn (and Sn) have a slight increase in both tissues, and Mg only in the muscles, but all nonsignificant. Fe and Ca in abdominal muscle increase going downstream, and in chitin the values are higher for both elements in the first two localities. In contrast, the values for Cu in muscle are higher in the other two localities downstream. Only the concentration of Hg in the muscles decreases significantly downstream, but there is no regularity in the exoskeleton.

At the second locality - Mokra Njiva, the lowest values were not detected in the meat of river crayfish, and the largest amount was registered for two metals: Hg and Si. In the chitin cuticle at site II, Al, Fe and Ca have the highest values. Chromium was detected only at this site. At site III, Duklov most, the highest amount was found only for Ni in abdominal muscle of noble crayfish. Potassium and Manganese increase downstream, properly in muscle, while in chitin they do not increase properly. The concentrations of Fe, Mn, Mg and K in abdominal muscle of crayfish from last sites was highest comparing to the other three regions.

DISCUSSION

To assess the health risk to the crayfish-consuming population, we investigated metals (18) in crayfish tissues (exoskeleton, abdominal muscle) in the upper part of the Zeta River. The Zeta River receives domestic and industrial untreated wastewaters from Nikšić city and surrounding villages during the last decades. In this study, the degree of metal accumulation in the tissues of crayfish samples collected from the Zeta River was investigated, because Crayfish may be used as an environmental indicator because their tissues tend to accumulate metals, including heavy ones (Anderson *et al.* 1997b). Almost all studies on the distribution of metals in crustacean tissues have shown that the hepatopancreas is the most important storage organ for trace elements and then gills or exoskeleton (Pourang and Dennis 2005, Alcorlo *et al.* 2006., Mistri *et al.* 2020, Fikirdesici-Ergen *et al.* 2019). The abdominal muscle has consistently been found in the literature to be the tissue containing the lowest concentration of metals (Anderson *et al.* 1997; Mackevičienė 2002, Naghshbandi *et al.* 2007, Kouba *et al.* 2010 Protosowicki *et al.* 2013), and our results also agree with this. According to Naghshbandi *et al.* (2007) only Zn concentrations were significantly higher in abdominal muscle of *A. leptodactylus* than carapace. Our analyses indicated that seven metals was accumulated in the greatest amounts in the exoskeleton (Table 1). Several metals were several times larger in the exoskeleton than in the muscles, e.g. Al, Fe, Sn. According by Fikirdesica-Ergen *et al.* (2019) Cu, Zn, Mn, Fe and Al are almost 10 times higher in exoskeleton than in muscle. Al is a metal abundant in nature and there was common belief that it does not have a negative effect on human health (Ranau *et al.* 2001). High levels of metals in the

exoskeleton may be due to the absorption of metals from water (Anderson *et al.* 1997a, b) or the fact that it is the route for both absorption and excretion of metals from the organism (Mackevičienė 2002). The opposite results for Cu, Zn and As were denoted by Stanek *et al.* (2017) for spiny-cheek crayfish and Mistri *et al.* (2020) for *Procambarus clarkii*. It is generally known that the chitin cuticle has a protective role, it is logical to expect that it contains the most heavy metals, which means that the cuticle, in addition to mechanical or physical protection (from injuries), protects internal organs and meat from harmful metals. Therefore, it has a special role in young crabs because they change their clothes five or six times during the first year (Reynolds, 2002).

Only two metals (Hg and Mg) were significantly higher in abdominal muscle of *A. astacus* from the River Zeta, than exoskeleton. In crayfish, mercury is accumulated largely in muscle (Simon *et al.* 2000; Loukola-Ruskeeniemi *et al.* 2003; Kouba *et al.* 2010) and according to Wiener *et al.* (2003) the mercury predominantly accumulates and in fish muscle. According to Kouba *et al.* (2010) and nickel accumulated largely in muscles and exoskeleton, respectively. Nickel accumulated approximately equally in both tissues of noble crayfish from Zeta river. The values of Hg and Mn were very high at each site studied (Hg - 97.1 to 33.1 mg, Mg - 253.5 to 200.1 mg) in muscle. Mercury is explicitly toxic substances, although their high concentrations are tolerated by organism. However, decapods are resistant organisms to environmental contamination, and even relatively high concentrations of metals (in this example Hg) are usually not responsible for their mortality (Kouba *et al.* 2010). High level some metals in the Noble crayfish from the river Zeta suggesting the pollution of the environment by heavy metals. The accumulation of metals in crayfish tissues is dose- and time-dependent, and therefore may be reflective of the levels of metals in the environment (Antón *et al.* 2000; Sánchez-López *et al.* 2004; Alcorlo *et al.* 2006; Allert *et al.* (2009). However, the measured values for Pb, Cd and Cr were below the threshold, thus suggesting limited contamination in the Nikšić area. No significant differences were found for the other trace elements content among the sampled tissues. With regard to the concentrations, the metals formed the following order in muscle: Mg > Hg > Ca > Si > Zn > Cu > Fe > As > Al > Mn > K > Na > Sn > Ni > Cr, and in exoskeleton: Ca > Mn > Fe > Si > Sn > Al > Cu > Zn > As > Hg > K > Na > Mg > Ni > Cr.

The observed order of increasing metal concentrations in crayfish organs is no consistent with their content in the lithosphere and hydrosphere provided by Information on the state of the environment in Montenegro for 2010, Ministry of Sustainable Development and Tourism 2010, Environmental Protection Agency of Montenegro). This shows that these arrays of metals are a consequence of pollution in this area. However, Iron, Mn and Al (in the Zeta river basin) are naturally abundant in sediments in Montenegro. Contamination can originate from a wide variety of anthropogenic sources such as disposal of high metal wastes in improperly protected landfills, land application of fertilizer, animal manures, sewage sludge, compost, pesticides and coal combustion residues

located in the Zeta river basin. According to Wuana and Okieimen (2011), most commonly found at contaminated sites are: Pb, Cr, As, Zn, Cd, Cu, Hg, and Ni. According to Bagatto and Alikhan (1987) the content of zinc in the body of a crayfish is naturally high. The concentrations of As, Cr, Hg and Ni in the present study were higher than the maximum permissible limits for human consumption established by Montenegrin legislation. Although no EU threshold limit for human consumption of crayfish muscle is established for Cu and Zn, other national regulations set maximum limits, e.g. for Cu at 20 mg kg⁻¹ w.w. in edible mass (Spain: Boletín Oficial del Estado 195, 15/8/1991), for Zn at 70 mg kg⁻¹ w.w. (U.S. Food and Drugs Administration), as reported by Alcorlo *et al.* (2006) and our results also agree with this. The content of these metals in crayfish organs varied depending on where they were caught. The highest metals content was detected in the downstream samples (3 and 4) close to main wastewater discharge point suggesting an anthropogenic contribution to total metals concentrations in muscle of crayfish from River Zeta. Only one metals - Hg were significantly higher on first and second sites in abdominal muscle of *A. astacus*, while crayfish collected upstream showed lower values for all heavy metals. The Zeta River receives domestic and industrial untreated wastewaters from Nikšić city and surrounding villages during the last decades. Noble crayfish does not have a large home range, hence migrations do not influence the level of metals accumulated in its tissues (Bohl 1999). Specimens are therefore representative of the locations in which they are caught.

Mercury concentrations were similar among sites. Based on a comparison, we found higher Hg concentrations with respect to those found by Finerty *et al.* (1990), Hothem *et al.* (2007) etc. from highly industrialized areas. We assume that the source of Hg pollution comes from medical waste that is uncontrolled discharged near the riverbed, or is derived from pesticides. The pollution of the environment by heavy metals is a result of various industrial activities and is also a multi-element problem in many areas of the world (Waisberg *et al.* 2003., John *et al.* 2008). The highest Al, As, Ni and Si concentrations in crayfish were found near the contaminated sediments at Zeta River. The high values draw attention to the land-based domestic and industrial inputs. The Zeta River receives domestic and industrial untreated wastewaters from Nikšić city and surrounding villages during the last decades. Agriculture takes important place for the economic value of the study area. Conservative fungicides that contain Cu are mainly used for fruits plantations and green-houses around the study area and composed fertilizer enriched with Zn and microelements fertilizer contains Mn are also widely used for farming around study area. Measuring Hg, Pb, Cr, As, Ni and Sn levels in crayfish from the Zeta river is important to demonstrate the potential risk to humans if the crayfish are consumed.

Content of some toxic metals in the muscle of Noble crayfish from River Zeta exceed the statutory limits for fish and crayfish intended for human consumption. Therefore, the crayfish from this region, in general, are not safe for human consumption. The average Hg values were higher than the limits of

Montenegro Official Gazette (2009) and according to EU limits (European Union Regulation). Because contamination of crayfish by heavy metals may pose a real risk to consumer, therefore, it is important to have knowledge on heavy metals levels in the tissues of crayfish used for food, and further investigation should be continued in future studies. Although other studies have shown that muscle tissue contains the lowest heavy metal levels compared with other tissues, muscle tissue is an important measure from a health view point since it is the most edible part of the crayfish (Anderson *et al.*,1997; Alcorlo *et al.*,2006). Environmental pollution by heavy metals is an escalating problem worldwide.

CONCLUSIONS

This study was carried out to provide information on heavy metal concentrations in noble crayfish which were consumed by local people and have commercial importance. Crayfish accumulated higher levels of heavy metals in the exoskeleton than in the muscle, consistently with previous studies. The levels of certain metals accumulated in the abdominal muscle exceeded the threshold values established by the EU (e.g. Hg) legislation. Measuring the accumulation of metals in crayfish tissues from the Zeta river is important to demonstrate the potential risk to humans if the crayfish are consumed. Our results also evidence that Noble crayfish living in the River Zeta are not suitable for human consumption. Because contamination of crayfish by heavy metals may pose a real risk to consumer, therefore, it is important to have knowledge on heavy metals levels in the tissues of crayfish used for food, and further investigation should be continued in future studies. To the legislators, we propose *A. astacus* for use as a bio indicator of heavy metals due to its ability to accumulate these environmental pollutants.

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PRELIMINARY RESULTS OF THE EFFECT OF ARTIFICIAL MYCORRHIZATION ON THE GROWTH OF SIBERIAN SPRUCE (*Picea obovata* Ledeb.) SEEDLINGS AND SOIL PROPERTIES

SUMMARY

In this study, we investigated the effects of artificial mycorrhiza application on soil properties and seedlings growth of Siberian spruce (*Picea obovata* Ledeb.) in the Novodolinsky nursery in Kazakhstan. Before transplanting, the roots of 3-years old seedlings were subjected to the mycorrhizal solution. The mycorrhizal strain includes mycelium of fungi of the genera *Suillus* Gray, *Boletus* Bull., *Paxillus* Fr., and *Cortinarius* (Pers.) Gray. The results showed that by applying the mycorrhizal activator, there were minor changes in the absorbed bases and soil pH. The value of phosphorus in the A horizon increased twice. The survival rate of the mycorrhizal seedlings is on average 2.4% higher than in the control. Significant differences were found in terms of the variables of the yellowing needles on seedlings, the formation of buds on young shoots, and the lignified stem of the seedlings. Particularly, in late August observation, mycorrhizal seedlings showed 14.2% more lignification. Due to the lignification, seedlings might be more resistant to stress factors such as drought, frost. These preliminary results are giving the information about adaptation to extreme ecosystems of the seedlings.

Keywords: Ectomycorrhizal fungi, mycotrophy, survival rate, symbiosis

INTRODUCTION

Preservation of environmentally favourable living conditions in various regions of the world directly depends on the rational and careful use of forest resources. Forest ecosystems of Central and Northeast Kazakhstan are one of the most important components of the Earth's biosphere, contributing to the ecological balance on the entire planet. Among the several categories of mycorrhizal fungi, ectomycorrhizal fungi is one of the major groups with

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arbuscular mycorrhizal in below-ground fungal communities in forests (Peay *et al.*, 2016). Ectomycorrhizal fungi are obligate mutualistic symbionts and hence rely entirely on carbon supply from host plants (Smith and Read, 2008). While they are abundant in root systems of herbaceous plants (Hiiesalu *et al.*, 2014), they are hosted also by diverse tree species (Liu *et al.*, 2015). Ectomycorrhizal fungi have a leading and play significant roles in forest community dynamics (Smith and Read, 2008) because they promote the dominance of the specific plant families e.g., Pinaceae, Fagaceae, Betulaceae, and Dipterocarpaceae (Tedersoo and Smith, 2013). Ectomycorrhizae are found in the upper layer, which mostly contains humus, rather than the lower layer of the soils which has the mineral-rich substances (Dogmus Lehtijarvi, 2007). The ectomycorrhizal fungi improve plant nutrition in exchange for carbohydrates (Smith and Read, 1997; Sebastiana *et al.* 2018). They have an important role in the intake of Zn, Cu, Mn, Fe, Ca, K and N, especially P, which is slow in soil intake. In addition, mycorrhizas increase the efficiency water use, as well as improve soil structure and protect the soil against erosion (Dogmus Lehtijarvi, 2007). Previous studies have been emphasized that the use of mycorrhiza seedlings in afforestation, rehabilitation and restoration works in marginal sites can significantly increase the success of the plantations (Arocena and Glowa, 2006; Qiang-Sheng and Ren-Xue, 2006; Bennett *et al.*, 2017; Kharuk *et al.*, 2019). This subject is particularly important in marginal sites that have extreme ecological conditions and under higher climate change influence, than in core populations (Barbati *et al.*, 2018). The environmental conditions on poor sites favour numerous biotic harmful factors including pathogens (Haavik *et al.*, 2015).

One of the main research and applied subjects is the study of ectomycorrhizal fungi, and their significance in forest nurseries, the development of methods for artificial inoculation of plants and ways to control the ecological potential of ectomycorrhizal associations. The importance of the mycotrophic way of feeding forest trees is expressed, mainly in improving growth. Equally important, seedlings with successful mycorrhiza formation can survive best after transplantation (Taylor *et al.*, 2016). The presence of mycorrhiza and its development is an essential indicator of their quality, since plants with trees and shrubs seedlings developed mycorrhiza better take root and grow, especially on poor soils (Jo *et al.*, 2018). This symbiosis partnership allows participating in the circulation of nutrients, optimizing plant metabolism, activating mineral nutrition, and inducing resistance to drought, salinization, heavy metals, and pathogens (Taylor *et al.*, 2016). There is a consensus that these plant-fungal associations have profound impacts on nutrient cycling and vegetation dynamics in ecosystems, particularly in temperate forests (Bennett *et al.*, 2017; Taylor *et al.*, 2016; Jo *et al.*, 2018). The study of ecology and physiology of ectomycorrhiza (EcM) is concentrated mainly in Europe, North America and Australia (Smith and Read, 1997; 2008; Read, 1999; Finlay, 2005; Polenov, 2013). On the territory of the Republic of Kazakhstan, the study of mycobiota and EcM macromycetes was carried out by Nam (1998), Abiev *et al.* (2000), and Abiev (2015). Applied

aspects of the mycorrhization were investigated by Meshkov (2010), what were the first studies in Kazakhstan. His research was not focused only to obtain four types of macromycetes into the investigated culture but also to develop a technology for their scaling and application in the form of mycorrhized compost for reforestation in Zailiysky Alatau (Meshkov *et al.*, 2009a; Meshkov 2010). However, some researchers emphasize that for normal development of trees, specialized strains of macromycetes fungi forming EcM is needed. In addition, particularly coniferous seedlings are not able to achieve adequate growth if they are excluded from mycorrhizal occurrence (Kais *et al.*, 1981; Alvarez and Trappe, 1983; Valdes, 1986).

Some studies have been carried out on Siberian spruce (*Picea obovata* Ledeb.) and ectomycorrhizae for different purposes. Some of these are as follows: Seasonal growth, dynamics of morphological and anatomical structure, and sugar content in ectomycorrhizal roots of Siberian spruce were studied by Tvorozhnikova *et al.* (2009); In another research; the diversity of fungal mantles of Siberian spruce ectomycorrhiza was investigated in two natural environmental gradients (the Visim State Nature Biosphere Reserve (Middle Ural; 380-685 m a. s. l.) and "Denezhkin Kamen" Reserve (Northern Ural; 305-800 m a. s. l.). As results of this research; Shannon's and Simpson's diversity indices of mantles types did not depend on the site altitude. It was revealed that the transformation of ectomycorrhizal diversity occurs in different ways in natural and technogenic ecological gradients (Veselkin 2010). In another study by Veselkin (2004) stated that changes in the anatomical characters of ectomycorrhiza in Siberian fir and Siberian spruce were studied in natural forests polluted with heavy metals (Cu, Zn, Cd, Pb, As, and Fe) and sulfur dioxide. As technogenic load increased, the total radius of mycorrhiza terminals and plant roots included in them increased in the organic horizon and decreased in the mineral part of the soil. However, Kharuk *et al.* (2019) emphasizes in their work climate-driven changes in boreal forest including Siberian spruce; however, little is known about its symbiosis with fungi. The aim of this research was to investigate the effect using the artificial mycorrhiza application on soil properties and seedlings growth of Siberian spruce.

MATERIAL AND METHODS

Study site and climate characteristics

The research was carried out in the Novodolinsky nursery (N 49° 42' 601'', E 72° 43' 096'') of the Kazan State University of Karaganda region of Kazakhstan.

Karaganda is about 554 m above sea level and the climate is cold and temperate. The climate classification is warm-summer humid continental climate (Dfb) according to the Köppen-Geiger climate classes. The coldest month is averaging below 0 °C (or -3 °C). The average annual temperature in Karaganda is 2.8 °C in a year, and the average rainfall is 310 mm (Table 1) (Kottek *et al.*, 2006; Peel *et al.*, 2007; URL1, 2020).

Table 1. Climate characteristics of Karaganda region (Year: 1982–2012) (URL1, 2020)

Climate characteristics	Month											
	January	February	March	April	May	June	July	August	September	October	November	December
Average temperature (°C)	-15	-14.7	-7.9	4.7	12.7	18.4	20.5	18	12.4	3.3	-6.2	-12.1
Minimum temperature (°C)	-19.5	-19.6	-12.7	-1.2	5.7	11.4	13.9	11.4	5.7	-1.8	-10.5	-16.4
Maximum temperature (°C)	-10.4	-9.7	-3	10.7	19.8	25.4	27.2	24.6	19.2	8.5	-1.9	-7.8
Precipitation (mm)	20	17	17	23	40	32	37	29	21	29	23	22

The climate of the nursery location is continental, in some years severe, with blizzards. The experiment was started on May 23, 2019 and observation time was 97 days. The main climate characteristics during the investigation (from July 23 to August 24, 2019) were observed. Namely, the average temperature was 21.6 ± 6.8 °C, the average daily maximum temperature was 27.3 ± 4.3 °C, and the average minimum morning temperature was 15.2 ± 3.4 °C, while the number of cloudless days was 15 days. The number of cloudy days was 16, and the average cloud cover was 3.9 points. The number of rainy days was 7 days, and the precipitation level was 217 mm. The average humidity for the observation period was $49.2 \pm 14.9\%$. The average level of the UV index was 4.5 ± 1.0 points (Anonymous, 2019).

Site preparation and mycorrhization

Three-years-old Siberian spruce seedlings from in the nursey of the Karaganda Region were used as research materials. The planting time was at the end of April and the beginning of May 2019. Before planting, the seedlings were stored under snow for 1 week. As morphologically homogeneous seedlings (Average height: 36 ± 8 cm) as possible were used in the research. Visual inspection of the seedlings showed that the overall condition of the seedlings is satisfactory, and no signs of wilting and necrosis were recorded. The buds were dormant, and the roots were moistened. Site preparations were performed using the MTZ – 80 tractors, and a 4-row sewing machine for the MTZ-82.1.8.

Before planting, the seedlings were subjected to the process of soaking the root system in a mycorrhizal solution using the following method: 1.1 kg of the mycorrhizal substrate (produced by Bio-Logica) was diluted in 10 liters of water with the addition of 200 g of agropelite (produced by Morov Agro). The substrate used is free-flowing, based on peat and living mycelium of fungi, immobilized on secondary aluminosilicates. Seedlings were exposed to the

mycorrhizal solution for 25 minutes. Planting of seedlings was carried out according to the generally accepted method of "Planting seedlings of forest crops in transplant section of forest nurseries" (Meshkov *et al.*, 2009a; Meshkov, 2010). The row spacing was 80 cm, while the distance between seedlings was 60 ± 10 cm. Planting depth was 15-20 cm.

Mycorrhiza was introduced into the root system at the same time as planting. The biological product called Mycorrhizal activator (produced by Bio-Logica) was also applied. This mycorrhizal activator contains active strains of living rhizosphere microorganisms and is grown based on natural material taken from the roots of a specific coniferous culture. The mycorrhizal activator included mycelia of fungi of the genera *Suillus*, [*S. variegatus* (Sw.) Richon and Roze 1888, *S. luteus* (L.) Roussel 1796, *S. sibiricus* (Singer) Singer 1945, *S. bovinus* (L.) Roussel 1796, *S. tridentinus* (Bres.) Singer 1945, *S. salmonicolor* (Frost) Halling 1983, *S. granulatus* (L.) Roussel 1796, *S. placidus* (Bonord.) Singer 1945], *Boletus*, [*B. satanas* Lenz, 1831], *Paxillus* [*P. involutus* (Batsch) Fr., 1838] and *Cortinarius* [*C. nemorensis* (Fr.) JE Lange 1940, *C. sp. sensu* NCL (1960)].

Analysis of soil samples

A detailed morphological description of genetic horizons was carried out according to standard methods (Rozanov, 2004). Three soil samples were taken from each depth (0-20 cm and 20-40 cm) and horizon. The soil samples were taken from 10-15 cm distance to the planting rows of seedlings. The following analyzes were performed: 1) Determination of the physical properties of soils (soil density (g/cm^3), the solid phase density of the soil by the pycnometric method, porosity (%) according to calculations, soil moisture by thermostat-weight method), determination of the granulometric composition of soils by the method of N.A. Kachinsk (Polupan, 1981), 2) Determination of physicochemical properties by potentiometric method, the amount of absorbed bases by the trilonometric method, humus (%) by the method of Tyurin (Tyurin, 1951), carbonate content by a gasometrical method, analysis of water extract (dry residue, anion composition: CO_3^{2-} , HCO_3^- , Cl^- , and SO_4^{2-}) and cations (Ca^{2+} , Mg^{2+} , Na^+ , and K^+) (absorbed base, mg/equiv per 100 g soil), mobile forms of phosphorus (mg/100 g soil) by the method of Machigin B.P (Sychev, 2000), exchange potassium (mg/100 g soil) on a flame photometer, pH, pedotransfer functions (PTF) (g/cm^3), 3) the samples were taken by a soil drill using the continuous column method every 10 cm to 100 cm to determine soil moisture and every 10 cm to 40 cm to determine the density, density of the solid phase of the soil and wet sieving aggregate content (%) of the soil, as well as in layers 0-20 cm and 20-40 cm to determine the amount of absorbed bases, humus (%) and nitrogen, phosphorus, and potassium (NPK) (Fly, 2004).

Seedling morphometric and phenologic characteristics

The following parameters of the seedlings were observed end of the vegetation period and measured by digital meters without removal from the soil:

1) Survival rate (SR-%), 2) terminal shoot length (TSL-mm), 3) lateral shoot length (LSL-mm), 4) the length of needles on the young shoot (LNYS-mm), 5) the yellowing needles on seedlings (YNS-pcs), 6) the formation of buds on young shoots (FBYS-pcs), 7) the number of seedlings with young shoots (NSYS-pcs), and 8) number of lignified stem (NLS-pcs). NLS has observed two times (Mid of July and end of August) in 2019.

The data of Siberian spruce seedling for the tested parameters were observed with three replicates. Among the measured characteristics of conifer seedlings “shoot length” and “length of needles” were observed. These indicators correlate with the degree of mycotrophy of coniferous seedlings (Ivanov *et al.*, 2014).

Statistical analysis

In the analysis of the obtained data, the t-test was applied for each measured parameter to compare the average values of the two data sets. The measured parameters were obtained with 3 repetitions and 30 seedlings in each repetition. For the data obtained, normal distribution compliance test checks were performed, and the necessary statistical transformations (arc-sin, logarithmic) were applied. All the analyses were performed using the SPSS program (version 11, IBM Corporation, Armonk, NY, USA).

RESULTS

The effects of artificial mycorrhization on soil properties

The obtained data present that the granulometric composition, i.e., the chestnut soils of the nursery, sandy loam is formed on sand. Sand fractions of 0.3-0.7 mm in size are found throughout the soil profile. Throughout the profile, the soil is loose with a sand granulometric composition. In the upper horizon A₁ of 0.2-17 cm of soil in the deposit, the content of physical clay is 19.2%, in the underlying horizons it gradually decreases in the parent rock and decreases to 9.24%, which can be confirmed by the sand granulometric composition of the parent rock. A similar change in the content of physical clay along the profile occurs in the soil of the experimental plot, where in the arable horizon of 0-26 cm the value of physical clay is 15.08%.

The maximum content of the fraction of physical clay is found (17.60%) in the B₁ horizon 26-36 cm per due to an increase in the silt fraction during washing and during irrigation. Further in the underlying horizons there is a decrease in physical clay, where in the parent rock it decreases to a minimum of 8.92% (Table 2). The humus content of the upper horizon A in control and in the experimental plot was same (1.18%). In the lower soil horizon of the control, B₁ 17-37 cm, is 0.74%, and in this horizon of the experimental site, B₁ 26-36 cm is 0.84%. The content of phosphorus in the all-soil horizons is very low however, the value of phosphorus in the A horizon has been found to increase 2-times thanks to the mycorrhizal activator compared to control (Table 3).

Table 2. Granulometric composition of chestnut soils of the nursery

Horizon and depth (cm)	The dimension of granule, % to dry soil								
	>3 mm	3-1 mm	1-0,25 mm	0,25-0,05 mm	0,05-0,01 mm	0,01-0,005 mm	0,005-0,001 mm	<0,001 mm	$\Sigma < 0,01$ mm
Control- Grassland without mycorrhization									
A ₁ 0,2-17	0.02	0.35	38.57	37.19	5.04	3.20	6.80	9.20	19.20
B ₁ 17-37	0.00	0.04	39.05	42.79	4.60	1.28	4.36	7.92	13.56
B ₂ 37-62	0.80	6.05	19.39	65.85	4.20	0.88	3.00	6.68	10.56
B _K 62-87	0.07	0.22	24.71	62.33	3.28	0.32	4.64	4.72	9.68
B _K C 87-106	0.10	1.50	35.64	50.40	5.96	0.28	2.64	5.08	8.00
C 106-124	0.30	0.92	33.50	53.78	3.48	0.48	3.20	5.56	9.24
Experimental plot with mycorrhization									
A 0-26	0.39	1.76	39.27	40.21	5.44	2.32	5.08	7.68	15.08
B ₁ 26-36	0.14	0.63	41.75	30.53	10.12	2.84	2.40	12.36	17.60
B ₂ 36-67	0.01	0.11	32.42	46.14	7.24	0.16	3.56	10.48	14.20
B _K 67-107	0.00	0.47	40.43	41.89	8.12	0.24	3.84	5.48	9.56
C 107-120	0.69	3.32	43.56	43.64	3.88	0.76	3.36	4.80	8.92

Table 3. Physicochemical properties of chestnut soils of the nursery

Sampling depth cm	Humus%	pH	Mobile mg/100 g soil		Absorbed base mg/equiv per 100 g soil			Absorption basis in% of the amount or capacity	
			P	K	Ca ²⁺	Mg ²⁺	Total	Ca ²⁺	Mg ²⁺
Control - Grassland without mycorrhization									
A ₁ 2-17 cm	1.18	6.6	0.40	22.20	7.70	1.60	9.30	82.79	17.21
B ₁ 17-37 cm	0.74	6.7	0.30	10.14	6.20	1.90	8.10	76.54	23.46
B ₂ 37-62 cm	0.53	6.7	0.20	8.71	5.80	2.15	7.95	72.96	23.04
Experimental plot with mycorrhization									
A _{max} 0-26 cm	1.18	7.0	0.80	32.30	7.35	1.80	9.15	80.33	19.67
B ₁ 26-36 cm	0.84	6.7	0.30	10.00	6.80	1.80	8.60	79.07	20.93
B ₂ 36-67 cm	0.59	6.9	0.20	5.00	4.10	1.80	5.90	69.49	30.51

The changes in the chemical properties of chestnut soils in the nursery were investigated with application of the mycorrhizal activator. The results have shown that when this treatment was applied to the soil, there were minor changes in the amount of absorbed bases and the reaction of the soil solution (Table 4).

The exchange ability of the soils is low due to the light particle size distribution. The amount of exchangeable bases ranges from 9.30-9.40 mEq per 100 g of soil in the upper layer of 0-20 cm. When the mycorrhizal activator is introduced in the soil, a slight change in the increase in the number of exchange bases occurs. With the option of using mycorrhizal activator in a layer of 0-20 cm, the number of cations increased. Changes of this value were also noted in the lower layer of this soil, where with the use of mycorrhizal activator was 8.90 mEq/100 g, and whereas this application was not the case- the sum of cations was 8.65 mEq/100 g. This value increased, which might be explained by more increased sorption process of the soil when using mycorrhizal preparation. In the layer of 20-40 cm of soil with the use of a mycorrhizal biological product, there has been an increase in the amount of cations by 0.25 mEq/100 g compared to the version without a biological product, but these changes are related to an increase in the proportion of Mg^{2+} cation in the soil adsorption complex. Ca^{2+} predominates in the composition of the absorbed soil bases in all cases, which accounts for 81.18-81.91% in the upper 0–20 cm layer, where in the cases by using the mycorrhizal biological product. The Ca^{2+} cation has been increased very slightly compared to control (Table 4).

Table 4. Changes in the chemical properties of chestnut soils

Sampling depth (cm)	pH	Absorbed base mg-eq per 100 g of soil			Absorbed base in% of the amount or capacity	
		Ca^{2+}	Mg^{2+}	Total	Ca^{2+}	Mg^{2+}
Control - Siberian spruce without mycorrhizal activator						
0-20	6.9	7.55	1.75	9.30	81.18	18.82
20-40	7.1	6.85	1.80	8.65	79.19	20.81
Siberian spruce by applying the mycorrhizal activator into the soil						
0-20	6.7	7.70	1.70	9.40	81.91	18.09
20-40	6.9	7.00	1.90	8.9	78.65	21.35

A negligible amount of the soil density has been decreased when compared to the control. Depending on the density of the solid phase in different variants of the soils, the porosity in the upper 0-10 cm layer has changed in the range of 51,03-51,66%. In the lower layer of 10-20 cm, these values vary from 50,99-51,76% (Table 5). According to porosity, these arable soils are rated as satisfactory. In the fallow variant, the upper soil layers are dense and have a porosity of less than 50%.

According to the structural analysis for wet sieving, in all variants of the soil layer 0-40 cm the number of water-resistant aggregates is higher than 51.06%, which indicates a satisfactory degree of structurality of the soils. In all variants using the biological product of chestnut soils, a slight increase in the aggregates is greater than 0.25 mm of soils by 0.78% in the 0-20 cm layer and 2.83% in the 20-40 cm layer (Table 6).

Table 5. Change of physical properties of chestnut soils

Depth of sampling cm	The density of the soil g/cm ³	PTF soil g/cm ³	Porosity %
Control - Siberian spruce without biological product			
0-10	1.18	2.41	51.03
10-20	1.25	2.55	51.76
Siberian spruce by applying the mycorrhizal activator into the soil			
0-10	1.16	2.40	51.66
10-20	1.23	2.51	50.99

Table 6. Change in water-resistant aggregates of chestnut soils

Depth of sampling cm	Wet sieving aggregate content, %				$\Sigma > 0.25$ mm
	1-2 mm	0.5-1 mm	0.25-0.5- mm	<0.25 mm	
Control - Siberian spruce without biological product					
0-20 cm	4.32	16.13	31.00	46.11	51.45
20-40 cm	5.03	14.25	31.78	47.39	51.06
Siberian spruce by applying the biological product into the soil					
0-20 cm	3.33	17.45	31.45	46.22	52.23
20-40 cm	2.45	18.79	32.65	45.11	53.89

The effects of artificial mycorrhization on seedling characteristics

The results show that the survival rate of the mycorrhizal seedlings of Siberian spruce is on average 2.4% higher than the control. However, there has not been found a statistically significant difference between control and mycorrhizal seedlings as to variables of SR (%), the number of the seedlings with blooming buds (pcs), and the number of seedlings with active shoots (%). A statistically significant difference between control and mycorrhizal seedlings was determined in terms of the NSYS variable. The fact that mycorrhizal seedlings have less yellowing in needles than control seedlings can be considered as a positive effect of artificial mycorrhiza application. It can be stated that the resistance of mycorrhizal seedlings increases thanks to artificial mycorrhization. In control seedling, the number of seedlings with young shoots (pcs) is higher in

both the terminal and the lateral branches (Table 7). Mycorrhizal seedlings have more buds in young shoots than control seedlings can be evaluated as a positive indicator. Some of the buds are potential shoots of the following year. This can be considered as a sign that mycorrhizal seedlings will form a stronger crown.

Table 7. Survival rate and key growth biometric indicators of Siberian spruce seedlings after mycorrhization

Treatment	Survival rate (SR) (%)	Number of seedlings with young shoots (NSYS) (pcs)		Terminal shoot length (TSL) (mm)	Lateral shoot length (LSL) (mm)	The length of the needles on the young shoots (LNYS) (mm)	Yellowing needles on seedlings (YNS) (pcs)	The formation of buds on young shoots (FBYS) (pcs)
		Terminal	Lateral					
Control	70.4	103	111	29.1 ± 10.15	21.0 ± 7.95	18.4 ± 5.28	152	0
Mycorrhiza applied saplings	72.8	83.33	93.33	29.07 ± 8.64	22.53 ± 6.69	20,8 ± 6.91	103.33	9.7
<i>t test, P level</i>	0.939 ^{ns}	5.502 ^{**}	7.489 ^{**}	0,021 ^{ns}	1,946 ^{ns}	2.396 ^{ns}	6.720 ^{**}	2.857 [*]

P significance level; ns: non-significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

There was no significant difference between control and mycorrhizal seedlings as the key growth biometric indicators such as terminal shoot length, lateral shoot length, and the length of the needles on the young shoots. However, significant differences were determined in terms of the variables of the yellowing needles on seedlings, and the formation of buds on young shoots (Table 7).

In the control treatment, the number of seedlings in which yellowing of the needles is observed is 86.4% of the total number of living seedlings. In experimental replications, this parameter was significantly lower at the level of $p < 0.01$ according to the control.

Table 8. The effect of mycorrhization on the process of lignification of the seedlings

Month	The number of lignified stems (NLS)		
	Control	Mycorrhizal seedlings	<i>t value, P level</i>
	%	%	
Mid of July	38,8	51,2	2,939**
End of August	42,9	57,1	17,391***

P significance level; ns: non-significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

The number of seedlings with lignified stem was observed twice in mid-July and at the end of August. In both periods, statistically significant differences were determined between control and mycorrhizal seedlings. Particularly, in late August observation, mycorrhizal seedlings showed 14.2% more lignification than the control treatment. This value would be reflected in the survival percentage of seedlings in the next years (Table 8).

DISCUSSION

Meshkov *et al.* (2009b) have emphasized that priority should be given to forest rehabilitation on burned areas and cut-over lands, including the ribbon-like relict pine forests of the Irtysh region, the Kazakh upland, plain forests of Kostanai Province. In addition, Meshkov *et al.* (2009b) and Sarsekova *et al.* (2016; 2020) recommended that in many parts of Kazakhstan, in the degraded forest areas, mycorrhizas should be used as a major improvement strategy. Such practical projects should be implemented as soon as possible.

In this research, the granulometric composition of the chestnut soils gradually decreased the content of physical clay, i.e., from the horizon A₁ of the soil to the parent rock. A similar change in the content of physical clay along the profile has occurred in the soil by applying the mycorrhizal product. In spite of the same humus content, it was found that there are minor changes in the amount of absorbed bases and pH by applying the mycorrhizal activator. The exchange ability of the soils was low due to the light particle size distribution. A slight change in the increase in the amount of exchange bases occurs either 0-20 cm or 20-40 cm by applying mycorrhizal activator. The Ca²⁺ cation has been increased very slightly by using the mycorrhizal product. However, the value of P in the A horizon has been found to increase twice due to the mycorrhizal activator. This result is very important. Cosgrove (1967) stated that in the boreal forest ecosystem, P is second only to N as a major limiting nutrient for plant growth and a major part of soil P, sometimes as much as 90%, is sequestered in organic compounds. The majority of P in boreal forest soils is present as organic P, and phosphatase activity is therefore significant for the ability of ectomycorrhizal fungi to obtain P in these systems (Haussling and Marschner, 1989). In addition, the soils of the nursery have a neutral, slightly alkaline reaction of the lower horizons. As a result of this study, it was found that the soil pH varies slightly in all studied options. The relationship between mycorrhizal fungi and soil acidity has long been discussed in the literature (Kalliokoski *et al.*, 2010; Pena *et al.*, 2017). Ectomycorrhizal fungi are important source of organic acids in soil (Griffiths *et al.*, 2009) and affect the pH of the rhizosphere (Smith and Read, 2008). Eremin and Popova (2016) emphasized that soils, where mycorrhizal biological products are applied under the seedlings, have led to a very slight decrease in the reaction of the soil solution in the studied depths. Mycorrhiza has been noted by Eremin and Popova (2016) to decrease the pH in the rhizosphere, because of the selective absorption of ammonium NH₄⁺ ions and the release of H⁺ ions. Smith *et al.* (2003) stated that the low effectiveness of root and mycorrhizal

functions in the cold soils is due to insufficient mineral elements supply. The results obtained in this study are consistent with the general results of previous studies.

A negligible amount of soil density has been decreased by using the biological product on the spruce seedlings and the soil beneath. Against it, a slight increase has been determined in the aggregates. According to Rillig and Steinberg (2002), the free mycelium of mycorrhizal fungi promotes the aggregation of soil particles and modifies the soil structure, which affects the general physical properties of the soil. Mycobiont hyphae are involved in the stabilization of soil micro aggregates by binding soil particles and the accumulation of organic compounds. Aggregation helps maintain a porous but stable soil structure and prevent erosion. However, these data are for a one-year investigation. Mycorrhization of seedlings did not lead to significant changes in soil properties within one growing season. Therefore, the expected positive results of this research will be more clearly observed in further stages.

The presence of mycorrhiza on the roots of seedlings, trees and shrubs and the degree of its development is an essential indicator of their quality. In fact, plants with developed mycorrhiza form better root system and growth, especially on poor soils. It was experimentally found that seedlings without mycorrhiza die or have low survival rate (9–12%), while plants with well-developed mycorrhiza are characterized by high survival rate (70–100%) (Meshkov *et al.*, 2009a). In this research, the survival rate of the mycorrhizal seedlings is found higher than in the control seedling. Research supporting this result was conducted by Gryndler *et al.* (2004), Ortega *et al.* (2004), Pešková and Tuma (2010). Gryndler *et al.* (2004), Pešková and Tuma (2020) highlighted that inoculation with mycorrhizal fungi has a positive effect on plants' survival rate and growth, and it increases resistance to various abiotic factors and biotic harmful agents.

In this research, significant differences were determined between control and mycorrhizal seedlings for the lignified stem of the seedlings in the periods of mid of July and late August. Particularly, in late August observation, mycorrhizal seedlings showed 14.2% more lignification than the control treatment. These results reflect that the survival percentage and growth of mycorrhizal seedlings would be better in the next years. Tvorozhnikova *et al.* (2009) emphasized that the formation of symbiotic complexes of Siberian spruce with mycorrhizal fungi favors this species tolerance to the conditions of cold climate. The studies by Qiang-Sheng and Ren-Xue (2006) showed that mycorrhized plants are most resistant to drought, they have higher evaporation and photosynthesis rates, lower leaf temperatures, and higher levels of dissolved sugars and starch (Selivanov, 1975).

CONCLUSIONS

It can be concluded that the most important value of mycorrhiza is that it protects the plant from the effects of adverse environmental factors and induces the plant's immunity to numerous stress factors. Particularly, mycorrhizal

application as a tool would play an even more important role for Kazakhstan, which has severe climatic and unfavorable ecological conditions, especially under the influence of climate change. Moreover, results of this research give us valuable information about adaptation of ecosystems to the extreme environment. According to the initial results of this research, it was found that artificial mycorrhization had positive effects on the survival percentage of seedlings and the lignification of the seedlings. However, in the long-term, the research will be able to give more reliable results for the practices of afforestation and forest regeneration in marginal areas.

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ALLELIC POLYMORPHISM OF K-CASEIN GENE (CSN3) IN THREE MONTENEGRIN CATTLE BREEDS

SUMMARY

Genetic polymorphism of κ -casein gene was investigated in three cattle breeds reared in Montenegro: Brown Swiss, Busha and Gray breeds of cattle. The DNA of these breeds was genotyped for polymorphisms at the kappa-casein gene by a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) essay. A 351 bp fragment of κ -casein was amplified and digested with HinfI restriction endonuclease. Three genotypes were identified (AA, AB and BB) with frequencies of 0.162, 0.501 and 0.356 (respectively) in Brown Swiss breed, 0.267, 0.501 and 0.233 in Busha breed and 0.236, 0.500 and 0.264 in Gray cattle breed. The frequencies of alleles A and B were 0.403 and 0.597 (respectively) in Brown Swiss, 0.517 and 0.483 in Busha cattle and 0.486 and 0.514 in Gray cattle. The genotyping of κ -casein alleles (A and B) is of practical importance, since B allele is in positive correlations with commercially valuable parameters of cheese yielding efficiency. The results of high frequency of B allele in Brown Swiss cattle confirmed a good performance of this breed in Montenegrin population. Results could be used for possible increasing the frequency of desired alleles and genotypes by including it in the breeding program for Brown Swiss breed as well as in the preservation program for Gray cattle and Busha as the important animal genetic resources in Montenegro.

Keywords: κ -casein, PCR-RFLP, Busha, Brown Swiss, Gray cattle

INTRODUCTION

Molecular biology and genomic selection techniques development have enabled to obtain more detailed information on the individual genome structure. The discovery of PCR-RFLP (*Polimerase Chain Reaction - Restriction Fragment Length Polymorphism*) generated renewed interest in the use of gene marker loci as an aid to selection programs and playing an increasingly important role as genetic markers in many fields of animal breeding. By the PCR-RFLP technique it is possible to determine the variability of milk protein fractions.

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Cow milk contains two classes of specific proteins, i.e., the group of caseins and the group of whey proteins. Caseins constitute about 78-82% of bovine milk proteins and subdivided into four main groups: α S1-casein, α S2-casein, β -casein, and κ -casein (Azevedo *et al.*, 2008, Smiltiņa and Grīslis, 2010, Hristov *et al.*, 2012).

Genetic variants of κ -casein have been extensively studied in cattle at the protein and DNA levels, and numerous alleles have been revealed (Tanaskovska *et al.*, 2016). κ -casein (κ -CN) is being determined by the gene positioned at the 6th bovine chromosome. The genomic DNA encoding the CSN3 milk protein is about 13 kb that is divided into 5 exons and intervening sequences (Azevedo *et al.*, 2008, Smiltiņa and Grīslis, 2010). Some authors reported fourteen polymorphic types of κ -CN (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J). The A and the B alleles are the most frequent among all the species of the genus *Bos* (Djedović *et al.*, 2015, Dokso *et al.*, 2014, Hristov *et al.*, 2012). The existence of the E allele in some dairy cattle breeds was reported in the papers of Soria *et al.* (2003), Caroli *et al.* (2010), Taha and Punan (1993).

The genetic variants of milk proteins are resulting as a consequence of substitution or deletion of amino acids within the polypeptide chain (El Rafey and Darwish, 2008). A and B variants of κ -casein differ in the amino acids 136 and 148. At position 136, threonine is replaced by isoleucine, while at position 148, aspartic acid is replaced by alanine, for A and B, respectively (Azevedo *et al.*, 2008, Deb *et al.*, 2014, Dogru and Ozdemir, 2009). The restriction endonucleases HindIII, HaeIII, and MaeII could be used for distinguishing κ -casein alleles (Soria *et al.*, 2003).

One of the most important effects of the milk protein polymorphisms on milk traits of economic importance is their relation to the technological properties of milk and cheese production. In most of the κ -casein studies, the association of the B allele variant with some quantitative and milk processing traits was identified. The B allele found to be associated with thermal resistance, shorter coagulation time, better curdles and micelles of different sizes, which are preferable in cheese making (Azevedo *et al.*, 2008, Dokso *et al.*, 2014). In majority of recent studies, a positive effect of κ -CN-B allelic variant on the share of casein and total milk proteins has been observed (Molina *et al.*, 2006, Djedović *et al.*, 2015). The cheese yield from cows with genotype BB is 10% higher than in cows with AA genotype (Deb *et al.*, 2014, Tanaskovska *et al.*, 2016).

Identification of the polymorphic gene variants of milk proteins associated with productive traits opens additional possibilities for improvement of production of different breeds (Djedović *et al.*, 2015). B allele of κ -casein gene is integrated into cattle breeding programs in many countries. In recent years, some countries started trading milk and dairy products with desirable kappa and/or beta casein as well as breeding animals with genotype for kappa and/or beta casein. In some countries the genotype for κ -casein is present in sire catalogues for a long time (Potočnik, 2015).

Cattle husbandry in Montenegro is the most important branch of animal husbandry and agriculture in general. Annual production of cow's milk is about 180 thousand tons. Breed composition of Montenegrin cattle population is quite unfavorable because various crossbreeds dominate, about 50% of the total population. In the last two decades share of high productive breeds Holstein and Simmental has been increased, at the same time Brown Swiss and Grey cattle sharply decreased, while autochthonous Busa breed is in risk of extinction.

The aim of this study was to identify the genetic variants of κ -casein in three cattle breeds reared in Montenegro for a longer period (Busha, Grey cattle and Brown Swiss) by use the PCR-RFLP technique. The studies of milk protein genes polymorphism and their application for the identification of individuals with desirable characteristics as well as for the selection and breeding programs is the future. Genotyping is very important for the evaluation of biodiversity and preservation of these cow breeds.

MATERIAL AND METHODS

Animals

Blood samples were obtained from 96 cows of three breeds: 30 samples were collected from Busha cattle 31 samples from Brown Swiss and 35 samples were collected from Gray cattle.

Busha is an autochthonous breed of cattle, which was the most dominant breed in Montenegrin cattle population till the seventies of XX century, (approximately 90%). In the second half of XX century, Busha breed had been crossed or just replaced by more productive breeds. Today, Busha is endangered breed, with less than 300 animals and because of that it is involved in the program of in situ conservation (Marković *et al.*, 2020).

The Gray cattle in Montenegro today is a local breed originated from Tyrolean grey breed. First time it was imported on beginning of XX century as well as after Second World War, till the sixties, and used for improvement of production traits of Busha cattle. This breed is very adaptable, resistant and very suitable for hilly mountain region of Montenegro. However, its population is permanently decreased and now its share in total cattle population is about 5% (Marković *et al.*, 2020).

Brown cattle in Montenegro originated from Brown Swiss breed, which is occasionally imported since the seventies of last century (Radonjić *et al.*, 2017). The population of the Brown breed in Montenegro participates by 12% in the total cattle population (Marković *et al.*, 2020).

DNA extraction from blood

Blood samples were collected from v. jugularis into vacuum tube K2EDTA (3ml). The blood samples were maintained at -20° C until used for DNA extraction. DNA extraction was performed using DNA isolation kit (ZYMO research, USA) according to manufacturer's instructions, while phenol-chloroform standard protocol used for some samples (Marković *et al.*, 2018). The quality of DNA was checked by Nano-Vue Spectrophotometer taking ratio of

optical density (OD) value at 260 and 280 nm. Also, the quality of the DNA samples was examined on 1% agarose gel.

Genotyping of kappa-casein gene by PCR-RFLP method

The κ -casein genotype was determined using PCR-RFLP. Amplification of 351 bp fragment of κ -casein was done using the polymerase chain reaction (PCR) with primers: forward (5'-TT TAT GGC CAT TCC ACC AA-3') and reverse (5'-ATT AGC CCA TTT CGC CTT CT-3') as reported by Dogru and Ozdemir (2009). The amplification was performed in 30 μ l of final volume with 100 ng of genomic DNA. The reaction mix contained 10xPCR buffer, MgCl₂ (1.5mM), dNTP nucleotides (100 μ M), 1 μ M of each primers and AmpliTaq Gold polymerase (0.5 U). Amplification was performed in Mastercycler Pro (Eppendorf) gradient programmed for initial denaturation at 94°C for 5 min (initial denaturation), followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 60 sec, and final extension at 72°C for 7 min. PCR products were checked for amplification by electrophoresis on 1% agarose gel, in parallel with 50 and 100 bp DNA marker (Đokić *et al.*, 2020).

For genotyping, PCR product was digested with Hinf I restriction enzyme which was used for the determination of κ -casein alleles. The total reaction mixture of 15 μ L comprised of 10 μ L of PCR product, 1.5 μ L of enzyme buffer, 5 U of restriction enzyme and 3.2 μ L of dH₂O. After digestion at 37°C for three hours the digested products were resolved by electrophoresis on 3% agarose gel in parallel with a 100 bp DNA marker. The genotype patterns were visualized under UV light by Quantum ST400. The genotypes of cattle κ -casein gene were identified on the base of restricted fragments size.

Statistical analysis

Allele and genotype frequencies for the genetic variants of κ -casein gene were analyzed by Hardy-Weinberg equilibrium calculator including analysis for ascertainment bias (Rodriguez *et al.*, 2009). The chi-square statistic used to check whether the populations were in Hardy-Weinberg equilibrium: <http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/kitest.htm>.

RESULTS AND DISCUSSION

Frequencies of genotypes and allele of κ -casein (CSN3) were detected in 96 individuals, in Brown Swiss, Busha and Gray cattle. PCR amplification of the 351-bp fragment of the κ -casein gene produced a clear and distinct band on the gel, which is of major importance for successful digestion step of PCR products (Figure 1). Digestion of the PCR products with endonuclease HinfI generated presence of three fragments (Table 1).

The genotype AB had highest frequencies of all genotypes in the investigated breeds: 0.501 in Brown Swiss and Busha and 0.500 in Gray cattle. The frequency of BB genotype was higher in Brown Swiss than in Busha breed and Gray cattle (0.356 and 0.233, 0.264, respectively).

According to χ^2 test, all breeds were in Hardy-Weinberg equilibrium, suggesting that the κ -casein gene in investigated population was not influenced by selection (Table 2). Higher share of observed AB and BB genotypes determined in Brown Swiss breed could be a consequence of use of homozygous bulls (semen) to "desirable" allelic polymorphic B form. The frequency of AA genotype of Brown Swiss breed was the lower (0.162), than for Busha and Gray cattle (0.267 and 0.236).

Many other authors (Taha and Punan, 1993, Dogru and Ozdemir, 2009, Dokso *et al.*, 2014, Djedović *et al.*, 2015) found that the frequency of the AB genotype in the Brown Swiss breed was higher compared to other genotypes, while Potočnik (2015) determined a higher share of BB genotypes compared to AA and AB in this breed. The higher frequency of BB genotype in Jersey cattle (0.772) with absent of opposite AA genotype is reported by Ren *et al.*, 2011.

Table 1. Expected /observed fragment sizes (in base pairs, bp) after restriction PCR products with *HinfI*

PCR Product (bp)	Restriction Fragments Sizes (bp) and determined genotypes		
	AA	AB	BB
351	131; 89	261; 131; 89	261; 89

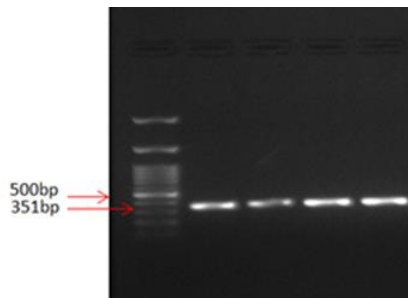


Figure 1. PCR products of κ -casein gene polymorphism visualised on 1% agarose gel

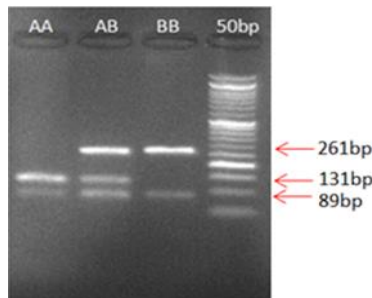


Figure 2. PCR-RFLP of κ -casein gene using *HinfI* on 3% agarose gel

Table 2. Frequencies of genotypes and alleles and Hardy Weinberg equilibrium test for κ -casein locus of Brown Swiss, Busha and Gray cattle breeds

Breed	N	Parameters	Kappa – casein genotypes			Allele frequencies		χ^2
			AA	AB	BB	A	B	
Brown Swiss	31	Ho	6	13	12	0.403	0.597	0.513
		He	5.04	14.91	11.04			
		Frequency	0.162	0.501	0.356			
Busha	30	Ho	8	15	7	0.517	0.483	0.000
		He	8.01	14.98	7.01			
		Frequency	0.267	0.501	0.233			
Gray cattle	35	Ho	7	20	8	0.486	0.514	0.723
		He	8.26	17.49	9.26			
		Frequency	0.236	0.500	0.264			

Ho: observed heterozygotes, He: expected heterozygotes on the basis of Hardy-Weinberg, χ^2 =chi-square value

Similar to our research, Djedović *et al.* (2015), Maletić *et al.* (2016), Brka *et al.* (2010) also obtained that heterozygote genotype of κ -casein (AB) was dominant in Busha breed. Only in the research of Ivanković *et al.* (2011) determined dominance of BB homozygote. Brka *et al.* (2010) found BC genotype at a low frequency (0.143), which was not found in Busha cattle in Montenegro.

For high productive Holstein Friesian breed some authors (Volkandari *et al.*, 2017, Tanaskovska *et al.*, 2016, Khaizaran and Razem, 2014, Dokso *et al.*, 2014, Ren *et al.*, 2011) determined higher frequency of AA genotype than AB and BB genotypes, what is similar to result obtained for Brown Swiss breed. Other authors, as Doosti *et al.* (2011), Gouda *et al.* (2013), Maletić *et al.* (2016) reported for Holstein breed the highest frequency of AB genotype κ -casein.

Different to our results, Djedović *et al.* (2015), Ivanković *et al.* (2011), Dokso *et al.* (2014), Taha and Punan (1993) identified very low frequency of BB homozygous of κ -casein gene for Simmental breed, predominantly lower compared to AA and AB genotypes. The frequency of BB genotype in the Simmental breed ranged from 0.096 (Djedović *et al.*, 2015) to 0.220 (Taha and Punan, 1993).

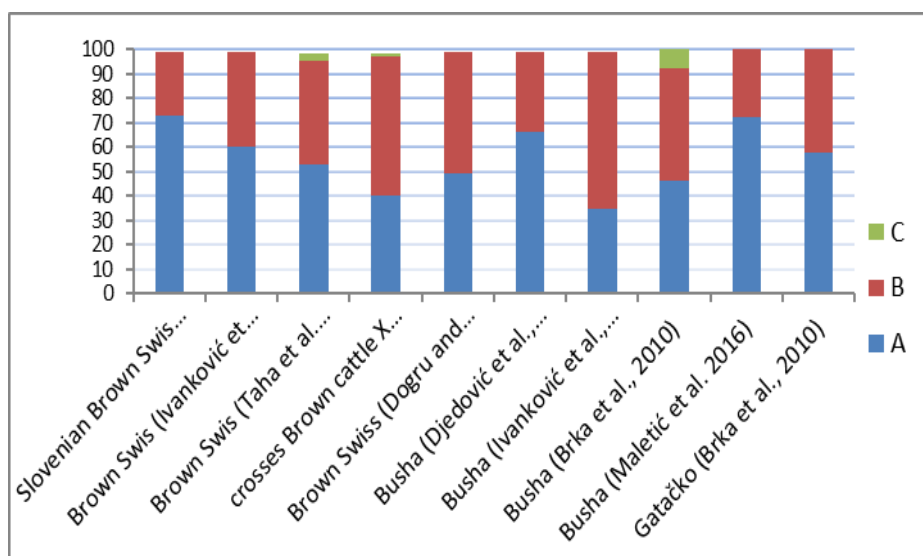
On the other side, the results of genotyping κ -casein of Czech Fleckvieh, Bulgarian Rhodopean Cattle, Shorthorn Rhodopean Cattle as old autochthonous breed (Kučerová *et al.*, 2006 and Hristov *et al.*, 2012), as well as results for Gatačko cattle reared in Bosnia and Herzegovina (Brka *et al.*, 2010) show strong

domination of the AB genotype, as it obtained for Gray cattle breed in Montenegro.

Table 3. The frequency of genotypes κ -casein in some cattle breeds reported by other authors)

Breed	Frequency of k-casein genotypes			Source
	AA	AB	BB	
Brown Swis (Slovenia)	0.069	0.387	0.544	Potočnik, 2015
Swiss Brown	0.279	0.480. AC 0.039	0.167. BC 0.034	Taha <i>et al.</i> , 1993
Crosses Brown cattle x American Brown Swiss	0.168	0.466. AC 0.016	0.334. BC 0.016	Taha <i>et al.</i> , 1993
Brown Swiss	0.194	0.602	0.204	Dogru and Ozdemir, 2009
Brown Swiss	0.392	0.449	0.159	Dokso <i>et al.</i> , 2014
Busha	0.417	0.500	0.083	Djedović <i>et al.</i> , 2015
Busha	0.444	0.555	0.00	Maletić <i>et al.</i> , 2016
Busha	0.206	0.294	0.500	Ivanković <i>et al.</i> , 2011
Busha	0.286	0.357	0.214. BC 0.143	Brka <i>et al.</i> , 2010
Gatačko*	0.308	0.538	0.154	Brka <i>et al.</i> , 2010

*Gatačko cattle is local name for population of Gray cattle breed reared in South East Bosnia and Herzegovina



Graph 1. Percentage of allelic CSN3 variants in cattle breeds (different authors)

In regarding allele frequencies of κ -casein gene for all three investigated breeds, slightly higher frequencies of A allele than B allele were determined. It was for Busha breed 0.517, Brown Swiss breed 0.597 and for Gray cattle 0.514.

Brka *et al.* (2010) obtained the similar ratio of A and B allele for Gatačko cattle (A - 0.58, B - 0.42) and Dogru and Ozdemir (2009) for Brown Swiss breed reared in Turkey. Very high frequency of allele A was reported by Potočnik (2015) for population of Brown Swiss in Slovenia (0.737) and Ivanković *et al.* (2011) for Brown Swiss in Croatia (0.607), Graph 1.

The higher frequency of A allele than allele B of Montenegrin Busha breed in our present study is in agreement with the earlier observations for Busha breed reared in other countries of Western Balkan, as it was reported for Busha in Serbia (Djedović *et al.* 2015, Maletić *et al.*, 2016) and for Busha reared in Bosnia and Herzegovina (Brka *et al.*, 2010). However, our results are not in accordance with the results for Busha breed in Croatia where lower frequency of A than B allele was found (Ivanković *et al.*, 2011).

Allele B of κ -casein was a favorable in cattle population because it significantly affected both milk and milk protein yield of Holstein and Native Iranian breed (Doosti *et al.*, 2011). Kučerová *et al.* (2006) also reported higher frequency of A allele (0.62) than allele B (0.38) in population of Czech Simmental cattle breed. Similar results for allele and genotype frequencies found in Czech Simmental (Fleckvieh) cattle population Kučerová *et al.* (2006), Holstein Friesian cattle in Macedonia and for some commercial cattle breeds in Croatia (Tanaskovska *et al.*, 2016, Ivanković *et al.*, 2011).

CONCLUSIONS

The results of PCR-RFLP analysis showed that three genotypes (AA, BB and AB) were found in the population studied for κ -casein gene. The results of very high frequency of B allele in Brown Swiss cattle implied that this breed should have a good performance in the milk traits related to the B allele of κ -casein gene. Hence, future studies should be focused on the association between cattle genotype and milk composition characteristics.

Determination of A and B allele frequencies in population of Busha and Grey cattle breeds could be used in the programs of selection and preservation, as important animal genetic resources in Montenegro, in order to increase frequency of desired alleles and genotypes.

Knowledge about genetic profile of breeds due to studied polymorphic variants of milk proteins is useful in further breeding development and economic valorization of cattle breeds, especially autochthonous and local ones.

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GEOLOGICAL HERITAGE MANAGEMENT AND PRESERVATION INSTRUMENTS: CASE STUDY OF THE MUNICIPALITY OF CONCEIÇÃO DA APARECIDA, MINAS GERAIS – BRAZIL

SUMMARY

Brazil is a country that has a vast territory and contains many natural landscapes features and immense variety of rocks, shapes and structures of great aesthetic and scientific value. Geosites are areas that have numerous geological potentials, where these characteristics are notable from a scientific, didactic or touristic point of view. In this sense, this work carried out a cataloguing of geosites in the municipality of Conceição da Aparecida, in the south of Minas Gerais state aiming at the geoconservation of the geological heritage, the balanced use of natural resources and the identification of geosites. The lands of the study area belong to several units of the crystalline basement entitled Campos Gerais Formation, Carrancas Group, Petunia Complex, Varginha-Guaxupé Complex and Andrelândia Complex composed essentially by gneissic rocks. The development of this work consisted in the construction of a qualitative base on the studied area from the analysis of topographic maps, the use of GIS and later field activity, laboratory analysis and the elaboration of the results. In this way, the relationship between man and nature can be evidenced from the proposals for geoconservation and the identification of these areas for future generations. Thus, the integrity of nature and the best quality of life for man will be maintained. The scientific and local community will be informed about the issues of geographic space, enabling investments and better management practices in the area.

Keywords: Geosites; Geodiversity; Geoconservation; Minas Gerais; Brazil

INTRODUCTION

Geodiversity consists of the variety of geological environments, phenomena and active processes that give rise to landscapes, rocks, minerals,

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fossils, soils, and other surface deposits that support the development of life. Based on this definition, it is possible to observe the relevance of studies on geodiversity, not only due to past changes, but also due to potential future impacts, adverse or favourable. Thus, the study and maintenance of geodiversity becomes increasingly important for the various areas of Geosciences (Brilha and Pereira, 2014; Brilha, 2016).

Geosites represent places of geological interest that have uniqueness due to their scientific, educational, cultural, and touristic value. Due to their strategic value, geosites must be identified, studied, and preserved for intrinsic scientific reasons or to serve humans with attributes of scenic beauty or supply natural resources such as water and mineral commodities (De Wever *et al.*, 2015).

Geomorphological mapping has tremendously evolved over the past decades (Frankl *et al.*, 2016) based approaches and the interpretation of stereoscopic aerial photographs. The mapping and study of geosites are recent in tropical regions and in southern Minas Gerais there are no considerable studies on this subject. Therefore, the absence of strategic information on geodiversity can lead to serious problems related to the socio-environmental impact of the absence of public conservation policies (Garcia *et al.*, 2018). Among them, we can mention the inappropriate use of natural resources, pollution and, in some cases, fatal accidents due to ignorance or lack of information and safety systems (Reverte and Garcia, 2016; Reverte *et al.*, 2019).

The set of geosites inventoried in the Taubaté Hydrographic Basin carried out by Reverte *et al.* (2019) identified 18 geosites. Therefore, they adopted geological categories to guide the identification of geosites during the inventory. The set of geosites contemplated have rock outcrops with high scientific and educational value and potential areas for the establishment of natural heritage. Then, Reverte *et al.* (2019) collaborated with strategic information for the stimulation of public policies for the conservation of geopatrimony.

The study of geodiversity is of interest for the development of an environmentally sustainable society, helps the emergence of environmental policies, the implementation and management of conversation units that address the uniqueness of geoheritages and contribute to the conservation of natural resources and encourage environmental education in its places (Brilha, 2016; Pitombeira, 2018; Reverte *et al.*, 2019).

Given the above, this work identified and classified the geosites of the Conceição da Aparecida municipality, in the Minas Gerais state, based on the proposal by Brilha (2016). The work also adapted the geosite identification and quantification method to tropical conditions and proposed measures to control and promote the environmental and socioeconomic sustainability of the area.

MATERIAL AND METHODS

The study area (Figure 1) covers the rural area of the Conceição da Aparecida municipality, south of the of Minas Gerais state – Brazil. The

municipality is located between the geographic coordinates 21°00'00" and 21°15'00" S and 46°25'00" and 46°00'00" W.



Figure 1. Location map of the of Conceição da Aparecida municipality, southern of Minas Gerais state - Brazil.

Located in the mesoregion of Alfenas in southern Minas Gerais, the municipality was founded on January 1, 1944 and has a population of 10,351 inhabitants according to the Brazilian Institute of Geography and Statistics – IBGE (IBGE, 2021). The main economic activity of the wider region and in the specific municipality is the traditional planting of coffee (Bolleli *et al.*, 2020; Tavares *et al.*, 2019). Tourist activities are concentrated during the festive periods of the city and in the hot season with ecotourism in the hydrographic region of Furnas Hydroelectric Power Plant Reservoir.

The geology of the study area consists of metamorphic rocks, which are mainly gneisses containing intercalations of shales in addition to metagabbros (Turbay and Valeriano, 2012). The main geological formations in the area are illustrated in figure 2, namely: the Campos Gerais Complex, Fortaleza de Minas Group, undivided Fortaleza de Minas and Andrelândia (Gaspar Junior *et al.*, 2010; SISEMA, 2019).

The digital map of soil classes were produced based on the Minas Gerais State Soil Map, scale 1:650,000 (Santana *et al.*, 2021; UFV *et al.*, 2010). The predominance of soils in the studied region is of red and yellowish Latosols and red yellow Argisols, with well-defined horizons and medium portion of humus

cover (UFV *et al.*, 2010). These have a generally sandy/clay texture, with a granular structure, with high porosity and well drained (Gaspar Junior *et al.*, 2010). Geomorphology comprises both quantitative and qualitative explanations of landscapes and landforms, as well as processes investigations and process interactions creating these forms in temporal and spatial scales (Curovic *et al.*, 2019; Hazbavi, 2018; Renschler and Harbor, 2002).

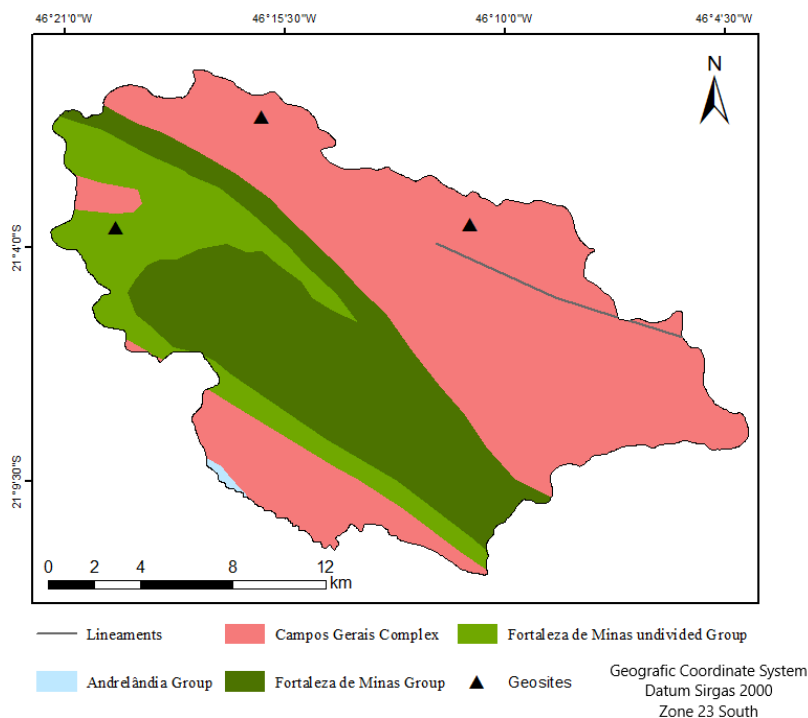


Figure 2. Geology Map of the of Conceição da Aparecida municipality, Minas Gerais state – Brazil. Data source: SISEMA (2019).

The local geomorphology consists of the Rio Grande Plateau and has altitudes below 1,000 meters, such as the Serras do Veado and Ibituruna, inserted in the context of seas of hills in the south of Minas Gerais (Silva, 2012).

The predominant morph climatic domain is the Atlantic Forest, with seasonal semi deciduous forest and the Cerrado (Savanna) associated with high altitudes. However, native vegetation has mostly been replaced by pastures and coffee cultivation (Scolforo *et al.*, 2008). The climate according to Köppen classification is humid subtropical (Cwa), with well-defined seasons, with a hot and humid between October and March and a hot and cold between April and September (Sparovek *et al.*, 2007; Aquino *et al.*, 2012).

At first, a bibliographical review on Geoconservation and Geosites was carried out, focusing on the relevance of the subject for science and society. Thus,

information regarding the physical aspects of the study area was collected and interpreted based on the satellite images, topographic maps and information available in the literature (Brilha and Pereira, 2014; Brilha, 2016; Reverte *et al.*, 2019). GIS and Remote Sensing tools are obvious complements to geoheritage research and conservation, due to their applicability across all facets of the research and conservation lifecycle (Sestras *et al.*, 2019; Williams, 2019).

Subsequently, fieldwork was carried out to collect data, which were analysed and synthesized using ArcGIS 10.5 geographic information systems.

Then, potential geosites were selected for educational, economic and tourist use. For this purpose, the field form adapted from Brilha (2016, Table 1).

Table 1: Geosite Classification Table

Location/Date:	Sample:
Coordinate:	Altitude:
Accessibility:	Conservation state:
Impacts:	Potential of use:
Interest:	Vulnerability:
Protection measures:	Physical aspects:

Source: Adapted from Brilha (2016).

Subsequently, the process of quantification of geosites was carried out following the proposal adapted of Brilha (2016). The quantification assigned grades to the geosites depending on their state of preservation. The criteria adopted for the quantification of geosites were: A – their intrinsic value; B - its potential for use and C - The need for protection (Table 2).

Table 2: Parameter for quantitative assessment

	Parameter for Quantitative Assessment		
	Intrinsic (a)	Land use (b)	Protection (c)
Parameter 1	A.1 Abundance	B.1 Potential use	C.1 Potential dangers
Parameter 2	A.2 Extension	B.2 Condition	C.2 Current situation
Parameter 3	A.3 Geological pr.	B.3 Accessibility	C.3 Mineral Potential
Parameter 4	A.4 Diversity	B.4 Proximity to villages	C.4 Fragility
Parameter 5	A.5 Conservation	-----	-----

Source: Adapted from Brilha (2016).

These grades range from one to five, with lower grades being areas with less relevance and high grades being more notable areas. Finally, the possibility of comparison and identification of the potential use of each element studied (Carcavilla *et al.*, 2009).

The vulnerability of geosites was classified as low, medium and high according to the criteria classified in the field by Table 1 and 2. Being: low when the sum of the criteria adopted is less than 15, medium when the values are from 15 to 19 and high when greater than 19. Then, possible land use and occupation actions for the identified geosites were presented and discussed, taking into account their vulnerability according to the method adapted from Brilha (2016).

RESULTS AND DISCUSSION

The analysis performed allowed to identify three main geosites with water, landscape and mineral interest, they are: Pedra Molhada Waterfall, Failure Waterfall and a Manganese Deposit. The summary of the quantification of the criteria adopted to classify the vulnerability of geosites is presented in Table 3.

Table 3: Summary of the analysis and classification of geosites.

GEOSITE	PARAMETER												
	INTRISEC					LAND USE				PROTECTION			
	A1	A2	A3	A4	A5	B1	B2	B3	B4	C1	C2	C3	C4
PEDRA MOLHADA WATERFALL	5	3	5	5	3	5	2	4	5	5	5	5	5
TOTAL				21			19			20			
CLASSIFICATION				HIGH			MEDIUM			HIGH			
FAILURE WATERFALL	5	2	5	4	3	5	5	5	5	5	5	5	5
TOTAL				19			20			20			
CLASSIFICATION				MEDIUM			HIGH			HIGH			
MANGANESE DEPOSIT	5	3	5	4	5	5	5	5	5	5	5	5	5
TOTAL				22			20			20			
CLASSIFICATION				HIGH			HIGH			HIGH			

Legend: A1: Abundance; A2: Extension; A3: Geological processes; A4: Diversity; A5: Conservation; B1: Potential use; B2: Condition; B3: Accessibility; B4: Proximity to Villages; C1: Potential Dangers; C2: Current Situation; C3: Mineral Potential C4: Fragility. Source: Adapted from Brilha (2015).

The Pedra Molhada Waterfall geosite (Figure 4 – item 1) is located between the municipalities of Conceição da Aparecida and Carmo do Rio Claro, approximately 20 km from the two urban areas, at coordinates 46° 14' 58" W and 21° 00' 22" S. Access to these geosites was classified as difficult due to the precarious situation of rural roads and their distance from the urban centre.



Figure 4a – Photographs 1: Pedra Molhada Waterfall; 2: Abandoned buildings from the old hydroelectric plant.



Figure 4b – Photograph 3: Water flow at the top of the waterfall and
Photograph 4: The pots formed by the abrasion of the rock.
Conceição da Aparecida municipality, Minas Gerais state - Brazil.

The area is in a good state of conservation; however, disordered tourism contributes to increasing the area's vulnerability. We suggest that the owner of the land where the geosite is located and public authorities promote conservation actions and environmental education. Thus, it is recommended to install environmental awareness boards, distribute educational pamphlets and install dumpsters on site. As demonstrated by Choque (2021) and Ruíz *et al.* (2021) environmental education allows strengthening the human relationship with the environment. This encourages specific actions for environmental conservation, such as reducing consumerism, recycling, and the proper use of natural resources. As a result, they directly impact the quality of ecosystems as they reduce exposure to imminent risks.

Due to the intense hydrological dynamics in the area, the gneissic rocks are highly altered, as shown in Figure 2 – items 3 and 4. At this point an old hydroelectric power plant is disabled. This improvement can be used as an operational basis for the development of activities related to scientific research, education and socio-educational practices combined with local tourism. This type of reconciliation of educational practices combined with leisure can generate satisfactory results in reducing environmental impacts due to the misuse of public space (Ruíz *et al.*, 2021).

Among the viable socio-educational practices, we can suggest the implementation of a natural history museum and a living space. In this way, it is possible to reconcile the conservation of the area with educational practices aimed at raising awareness about the use and conservation of natural resources and the sustainable economic development of local communities.

The second Fault Waterfall geosite is illustrated in Figure 5 and is located 15 km from Conceição da Aparecida at the coordinate $46^{\circ} 10' 54''$ W and $21^{\circ} 3' 33''$ S. The site is highlighted due to a change in relief caused by a geological fault (Figure 5). Therefore, it is a point of interest for scientific development purposes due to its geological condition considered didactic for the teaching of geosciences.



Figure 5. Fault Waterfall. Photograph 1: Waterfall and 2: Fault plane with stretched feldspar crystals, which suggest a geological fault site. Conceição da Aparecida municipality, Minas Gerais state - Brazil.

The area was classified as high vulnerability due to the presence of the geological fault. It is worth noting that it is inactive. In this location, constructions of any origin should be avoided, as instability in the area can vary. Therefore, access to this point must be controlled. Misuse of this site can compromise the quality of surface and groundwater. Thus, the potential for using this geosite is scientific and didactic due to the complexity of the elements that make up the landscape. If it is decided to use it for tourist purposes, it is necessary to establish measures to protect users, such as trained personnel, security measures and adequate cleaning.

The next geosite was called Manganese Deposit (Figure 6) and is 21 km from the urban perimeter of Conceição da Aparecida at $46^{\circ} 19' 45''$ W and $21^{\circ} 3' 33''$ S. Access is difficult through dirt roads precarious. The use of the local soil is for coffee cultivation, being remarkable the absence of native forest in the place. The exposure of soil and rock to climatic action favours soil losses due to water erosion as pointed out in the literature (Renard *et al.*, 1997; Prochnow *et al.*, 2016).



Figure 6. Manganese deposit exposed in a coffee cultivation. Conceição da Aparecida municipality, Minas Gerais state - Brazil.

The main rocks in the area have the presence of manganese ore. Manganese is an important element in the steel industry, as a deoxidizing agent; due to this factor its presence generates economic interest (Afonso, 2019). This manganese deposit from Conceição da Aparecida extends for approximately 5 km, containing minerals such as quartz, manganite, pyrolusite, psilomelane and cryptomelane.

At the Manganese Deposit geosite, since it is a mineral extraction point, proposals and projects for land use and occupation should be more restricted. The area is exposed and therefore vulnerable to environmental impacts such as soil loss due to water erosion. Thus, the move away from coffee cultivation and the recovery of native forest are essential to promote the sustainability of the use of the geosite.

Brazil has enormous geotouristic potential and favourable conditions to fully develop this activity, in order to enjoy the social benefits it can offer. One of the main benefits is to allow tourists to know the geological heritage that makes up the geotouristic scenario, leading the community to value it and, consequently, promote its geoconservation in a sustainable way (Henriques *et al.*, 2011).

CONCLUSIONS

The adaptation of the method of Brilha allowed us to objectively point out the elements that made it possible to classify the vulnerability of a geosite to environmental impacts in conditions of a tropical region.

The geosites covered in this work were classified as medium and high vulnerability. This classification was due to its intrinsic factors such as land use and occupation, presence of pollution and its conservation status.

The results allowed pointing out actions that aim to maintain the environmental balance in the use of geosites. Among the possible actions to maintain the sustainability of the geosites were: reforestation, establishment of projects aimed at reconciling environmental conservation and socioeconomic development.

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IN VIVO EFFECT OF *Origanum vulgare* ESSENTIAL OIL ON MICROBIOLOGICAL QUALITY OF RAW MILK FROM SUBCLINICAL MASTITIS AFFECTED COWS

SUMMARY

The study aims to test under in vivo condition the effect of *Origanum vulgare* essential oil (OEO) on the bacteriological quality of raw milk from cows affected by subclinical mastitis. The tested oil extracted from Algerian endemic oregano collected in Setif province (eastern Algeria) was characterised as carvacrol-chemotype using gas chromatography-mass spectrometry (GC-MS). The in vitro inhibitory effect of diluted OEO was tested on control reference strains. *Staphylococcus aureus*, ATCC 700699 methicillin-resistant seemed more susceptible to OEO than ATCC 25923 methicillin-susceptible. The in vivo trial was conducted on lactating and non-pregnant cows selected according to their California mastitis test scores. The microbiological analysis was applied for 100 units of raw milk samples collected before and three days after the twice a day topical application of OEO diluted with vegetable oil (5%). Results showed the only presence of total aerobic mesophilic bacteria (TMAB) and coagulase-positive staphylococci (CPS) in all samples with values close to the critical limit. After treatment, a significant decrease ($P < 0.001$) in the mean count of TMAB and a non-significant decrease in the number of CPS ($P > 0.05$) were noted. Results indicate that aromatherapy is a promising approach for improving udder health and raw milk microbiota.

Keywords: Carvacrol, Essential oil, Mastitis, Milk, Oregano, *Staphylococcus*

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INTRODUCTION

On a global scale, Algeria is a major importer of milk powder and derived products with a global bill estimated in 2018 at US\$1.40 billion, thus occupying second place behind cereals. The efforts and subsidy schemes put in place by the State over the past few decades with the aim to increase the production of raw milk and above all to encourage the emergence of a real agro-food industry (Meklati *et al.*, 2020), have come up against the difficulty posed by this raw material which remains complex and altered.

Few studies carried out in Algeria on the microbiological quality of raw milk samples taken individually on the production site have been published. The low hygienic bacteriological quality that exceeds the critical threshold was described in a study carried out on farms in the Tiaret region. The authors found the presence in the analysed samples of TMAB greater than 105 colony forming unit per millilitre (CFU/ml) (81.2%), faecal coliforms (18.06%), *Staphylococcus aureus* (81.93%) and faecal streptococci (80.64%) (Ghazi and Niar, 2011). This bacteriological quality was influenced in a study carried out in the Metidja region by the size of the dairy farm and the practice of udder disinfection (Hakem *et al.*, 2012).

Subclinical mastitis is one of the causes of the deterioration of the quality of raw milk collected from the farm often due to infections by potentially pathogenic germs, namely coagulase-positive staphylococci, enterobacteria (*E. coli*) and streptococci (FAO, 1989). However, the emergence of mastitis due to minor germs, especially coagulase-negative staphylococci, has been reported (Pyörälä and Taponen, 2009). In eastern Algeria, *Staphylococcus aureus* has been considered one of the major pathogens causing subclinical bovine mastitis (Zaatout *et al.*, 2020). It represents one of the germs often implying the use of antibiotic-based applications, but their efficacy remains low (10-30%) due to the potential of resistance of these bacteria (Gomes and Henriques, 2016). In addition to the risk to public health linked to the toxi-infectious potential of this germ, one of the consequences of this pathology is the marketing of milk with low industrial value due to the presence of antibiotic residues (Sachi *et al.*, 2019). As a result, the use of essential oils as a possible alternative therapeutic approach against mastitis to solve the problem of antibiotic resistance has been the subject of several *in vitro* experiments, but their properties have been very little tested *in vivo* on livestock (Baskaran *et al.*, 2009; Mushtaq *et al.*, 2018; Alagawany *et al.*, 2020; Gupta *et al.*, 2020). These studies have confirmed the efficacy of the antibacterial activity of the compounds of these oils on germs isolated from mastitis milk, particularly the phenolic compounds, including carvacrol. Its use as a feed supplement compound to improve animal health was reviewed by Sharifi-Rad *et al.* (2018). This phenol is generally present in significant percentages in Lamiaceae that grow spontaneously in North Africa (Algeria, Tunisia), especially *Origanum vulgare* (Sari *et al.*, 2006; Béjaoui *et al.*, 2013; Ali *et al.*, 2020; Nabti *et al.*, 2020).

The present study involves an in vivo trial preceded by an in vitro test on reference control strains, a first in Algeria at our level of knowledge, in order to test the effect of the essential oil of *Origanum vulgare* on the quality and bacteriological composition of the raw milk of cows exposed to the risk of subclinical mastitis. This approach aims to contribute to the development of new therapeutic option for bovine mastitis in accordance with the “One Health” concept.

MATERIAL AND METHODS

Essential Oils origin

The essential oils used in the study were obtained after steam extraction of the dried aerial parts (leaves and flowers) of *Origanum vulgare* subsp. *Glandulosum* (Desf.), an aromatic medicinal plant endemic to Algeria (Sari *et al.*, 2006). The plant material was collected during the flowering period in June 2019 in the mountains of Amoucha located in Sétif province in eastern Algeria (Fig. 1). The taxonomic identification of oregano specimen was made by Dr Samia LAKEHEL (Badji Mohktar University of Annaba, Algeria).

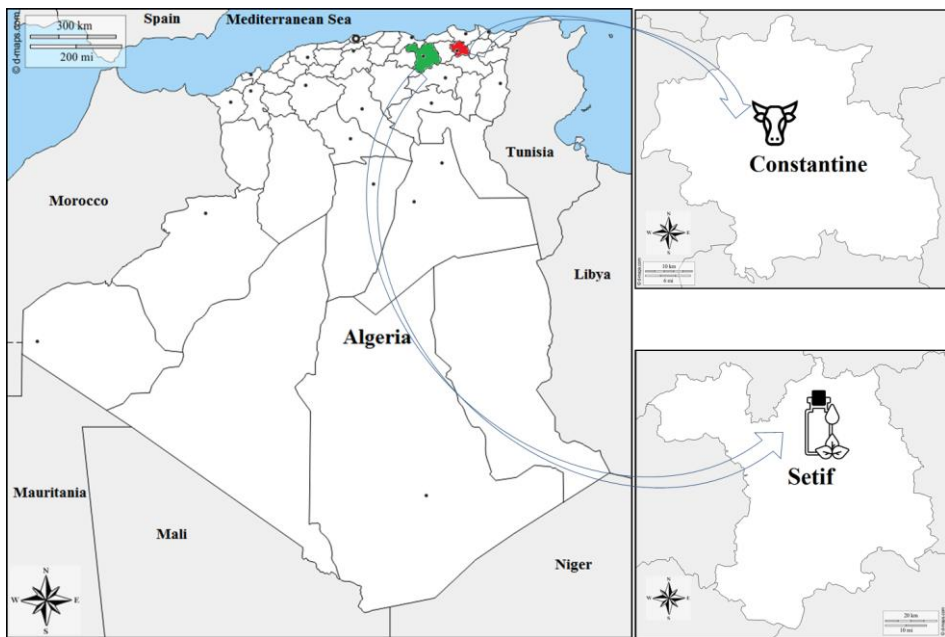


Figure 1. Location of the oregano collection region (Setif) and the study area of in vivo trial (Constantine)

Characterisation of oregano essential oil used in the clinical trial

The chemical composition of OEO to be used in the in vivo test was identified by gas chromatography-mass spectrometry (GC-MS). The analyses were carried out on an HP 6890 gas chromatograph coupled to an HP 5973 mass

spectrometer (Agilent, CA, USA) using HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). GC-MS spectra were obtained using the following conditions: injected essential oil volume was 1 µl in splitless mode; injection temperature was 250 °C; helium was the carrier gas at a flow rate of 0.5 ml/min; oven temperature program (60 °C for 8 min, increased at a rate of 2 °C/min to 250 °C, then held at 250 °C for 10 min). The mass spectral database used for essential oil component identification was Wiley 7N and NIST 02 (National Institute of Standards and Technology, USA).

In vitro efficacy of several dilutions of oregano essential oils on control reference strains

Control reference strains were used to evaluate the effectiveness of the oregano essential oils in inhibiting bacteria growth. *Staphylococcus aureus* ATCC 700699 (Mu50) applied in drug discovery was used as the reference control for methicillin-resistant (MRSA) strains. *Staphylococcus aureus* ATCC 25923 applied in the examination of dairy products and susceptibility disc testing was involved to control methicillin-susceptible (MSSA) strains. They were grown after spreading on Chapman's agar and incubated at 37 °C for 24 hours. After growing, a few well-isolated colonies of bacteria were taken and diluted in sterile physiological saline, then homogenised until an opacity equivalent to 0.5 units of the Mc Farland scale was obtained. Afterwards, 1 ml of inoculum was deposited and inoculated over the entire surface with Mueller-Hinton Agar (MHA) previously poured into a Petri dish.

The susceptibility of the reference strains to OEO diluted in the vegetable oil (VO), used both for in vitro and in vivo test, at the habitual (1%), recommended (5%) and double recommended (10%) concentrations for topical use (Franchomme *et al.*, 2001) was evaluated by measuring the diameter of the inhibition zone (aromatogram). For this purpose, 6 mm paper discs impregnated with 10 µl of each of the three doses mentioned above (1% , 5% , 10%) were used according to the disc-diffusion agar method for the evaluation of the activity of topical antimicrobial agents as described by Glasser *et al.* (2010).

The experiment was repeated for each dose and the data were presented as the mean and range of inhibition zone diameters. A threshold value of 8 mm was used to classify the bacteria tested as sensitive to the topical antimicrobial agent (Holder and Boyce, 1994). Also, the vegetal oil diluent without OEO was used as a positive control. A disc impregnated with 10 µl pure vegetable oil (100%) was applied on the MHA to check the sensitivity of the strains tested to the essential oil vehicle. Furthermore, a diffusion control test was performed using dimethyl sulfoxide (DMSO) as essential oil vehicle at the same tested concentrations.

In vivo assay of OEO effect

Study area: The clinical trial was carried out in March 2020 on a pilot dairy farm located in the province of Constantine (Figure 1) in north-eastern Algeria (36° 16' 17.1 "N 6° 40' 11.9 "E). This farm was chosen based on its

prevalence of subclinical mastitis that we have previously assessed (Hamlouli *et al.*, 2019).

Animals: The studied cows were selected from a herd of 85 dairy cows based on their California mastitis test (CMT) score. These females of Prim'Holstein breed, aged two to six years, were in lactation (post-partum period) and not pregnant. They had not previously received any antibiotic-based treatment.

Essential oil application: In the selected cows, the OEO oily solution recommended dose (Franchomme *et al.*, 2001) for topical use (5%) was applied twice a day, after morning and evening milking, by skin massage on the selected quarters for three successive days.

Sampling: A total of 20 raw milk samples were taken individually from 10 quarters, 5 affected (high somatic cells score) and 5 healthy (normal somatic cells score). They were taken twice, 10 samples before the application of the essential oil of oregano (day 0) and 10 samples 3 days later, each time keeping the same quarter of origin for each sample. The samples were taken aseptically, in compliance with the hygiene rules required for microbiological analysis, in sterile tubes labelled with a code linking it to the mammary quarter of origin. These samples were kept cold and sent to an analysis and quality control laboratory.

Raw milk analysis

CMT test: To screen the individual quarter milk for somatic cells, the CMT test was done before milking at the beginning, before the application of the essential oil, and at the end of the trial. CMT scores ranging from 0 (negative) to 4 (strong positive) were recorded according to the interpretation of test results described by Lévesque (2004).

Microbiological analyses: Repeated microbiological analyses (n= 5 units) were performed for each sample in a quality control laboratory according to the methods described in the Official Journal of the Algerian Republic for counting of: Total mesophilic aerobic bacteria (TMAB) on the Agar medium, with incubation at 30 °C for 72 hours. Coagulase-positive staphylococci (CPS) by plating 1 ml of raw milk on Baird Parker agar with incubation at 37° ±1 °C for 24-48 hours. Thermotolerant coliforms using lactose agar with incubation at 30 °C ± 1 °C for 24 hours ± 2 h. *Salmonella* on Hektoen agar, with storage in an oven at 37 °C for 18 to 24 hours. *Listeria monocytogenes* via Oxford agar. Incubation was carried out at 37 °C for 24 to 48 hours.

Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics 26 software. The comparison of the cytological and microbiological values with the accepted standard criteria was made through a Student 't' test. The Z test using the Bonferroni correction was performed for comparison between prevalence of groups categories formed according to levels of TMAB and CPS counts and time of treatment (before and after).

The linear model was applied to highlight the fixed effect of the time factor reflecting the oregano essential oil effects on the cytological (CMT scores and

ASC) and microbiological parameters (bacteria count and proportion to the total mesophilic flora). These parameters were considered as dependent variables. Also, the pairwise comparison t-test was used to compare the results of the affected and healthy quarters before and after treatment. A significance threshold of 0.05 was retained for all the tests.

RESULTS AND DISCUSSION

Oregano essential oil characterisation

The result of the GC-MS analysis of *Origanum* essential oil showed that the prevalent compound is carvacrol (39.27%) followed by thymol with 25.83% (Table 1 and Figure 2). This composition is similar to those reported in subsequent studies carried out in our study area (Sétif) on the same species, *O. vulgare* L. subsp. *Glandulosum*, which showed the presence of 04 major compounds thymol, carvacrol, p-cymene and γ -terpinene with the majority of samples belonging to both chemotypes, carvacrol and/or thymol (Sari *et al.*, 2006; Khalfi *et al.*, 2008). The predominance of carvacrol recorded was described in a recent study on samples of the same species collected in the Bougaa region of Sétif with a percentage of 26.29% (Ali *et al.*, 2020). Considering the endemic character of this plant in North Africa, a study in the Mediterranean Phyto-region, North-East Tunisia, showed that carvacrol with a value ranging from 61.08 to 83.37% was also the main compound of this *Origanum* species (Béjaoui *et al.*, 2013). On the other hand, in the same region, from data published in 2020 (Nabti *et al.*, 2020), carvacrol was present in a small percentage (2.8%) with thymol as chemotype (56.3%) in the chemical composition of the oil extracted from the same species. However, these authors (Nabti *et al.*, 2020) found a dominance of carvacrol (59.6%) in a region bordering ours (Bordj-Bou Arreridj) for the oil of the species studied.

In vitro effect of oregano essential oils on control reference strains

The results of the aromatogram (Table 2) show that the highest diameters of inhibition were obtained with the solubilisation of the oil in dimethyl sulphoxide (DMSO) for the two strains ATCC MU 50 and ATCC 25923, for a concentration of 10% with a diameter of 31.72 mm and 17.42 mm respectively. The efficacy of this oil against *Staphylococcus aureus* ATCC 25923 was also found by Bouhaddouda *et al.* (2016) who obtained a high inhibition diameter of 51.83 ± 2.56 mm using 5 μ l of undiluted essential oil of *O. vulgare* L. ssp. *glandulosum* (Desf.) Ietswaart collected from the province of Guelma (northeastern Algeria) with para-cymene (25.615%), thymol (23.129%), carvacrol (20.321%) as the main components using the agar disc method. It appears that the effectiveness of the essential oil is dependent on the diffusion ability of the vehicle (DMSO or vegetable oil). For both vehicles, MRSA were more susceptible to OEO than MSSA strain with a largest diameter obtained at 10% concentration. The 5% concentration seemed to be the limiting dilution among those tested allowing an inhibitory effect.

Table 1. Chemical composition of the tested oregano essential oil

Peak Number	Compounds	Time retention (min)	Peak area percentage (%)
1	β -Thujene	9.72	0.31
2	α -Pinene	10.10	0.29
3	Camphene	10.96	0.04
4	β -Pinene	12.69	0.06
5	Octen-3-ol	13.33	0.26
6	3-Heptanone	13.49	0.17
7	β -Myrcene	13.77	0.72
8	α -Phellandrene	14.57	0.11
9	3-Carene	14.92	0.04
10	α -Terpinene	15.45	1.27
11	p-Cymene	16.29	12.27
12	D-Limonene	16.39	0.68
13	γ -Terpinene	18.74	13.58
14	2-Hepten-4-ol	19.23	0.08
15	(+)-4-Carene	20.49	0.05
16	nd	21.48	0.03
17	Linalool	21.73	0.51
18	Terpinen-4-ol	27.11	0.25
19	α -Terpineol	28.25	0.15
20	Estragole	28.65	0.31
21	Thymol methyl ether	31.11	0.13
22	Isothymol methyl ether	31.74	0.24
23	Thymol	36.80	25.83
24	Carvacrol	37.72	39.27
25	Caryophyllene	43.20	1.11
26	α -Caryophyllene	45.31	0.03
27	β -Bisabolene	48.84	1.01
28	β -Sesquiphellandrene	49.68	0.22
29	α -Bisabolene (Z)	50.81	0.41
30	Caryophyllene oxide	53.11	0.28
Not-identified compounds			2.7
Total identified compounds			97.30

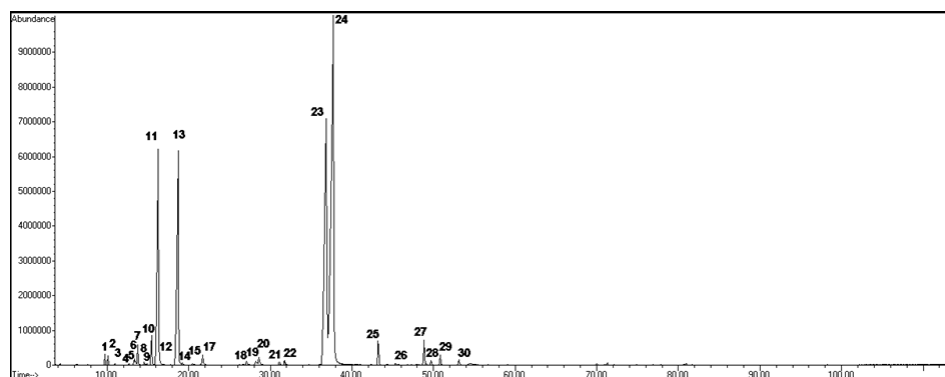


Figure 2. GC-MS Chromatograms and peak number of oregano essential oil compounds.

No control disc impregnated with vegetable oil alone inhibited the growth of the two reference strains. Regarding the 8 mm susceptibility threshold used to test the antimicrobial effect of the solutions for topical use (Holder and Boyce, 1994), The inhibiting effect was only obtained with the OEO diluted at 10% in vegetable oil. The susceptibility of ATCC 700699 strain to the essential oil of *Origanum vulgare* diluted to 10% was also found in a recent study by Cabrera-Contreras *et al.* (2020). These authors noted the absence of growth of this MRSA strain when it was inoculated on culture medium in mixture with essential oil undiluted or diluted in coconut oil at 10% , 1% , 0.5% and 0.25% (Cabrera-Contreras *et al.*, 2020). However, this adopted antibacterial activity test, which is different from our agar diffusion method, did not allow the MIC and inhibition diameter to be calculated.

Table 2. Inhibition effect of oregano essential oil on control reference strains at several dilutions

Reference strains	OEO vehicle	Diameter (mm) of inhibition (mean \pm SD) for OEO dilutions		
		1%	5%	10%
<i>Staphylococcus aureus</i> (ATCC [®] 700699 TM)	VO	3.65 \pm 5.16	7.03 \pm 0.06	8.84 \pm 0.21*
	DMSO	9.22	25.91	31.72
<i>Staphylococcus aureus</i> (ATCC [®] 25923 TM)	VO	3.13 \pm 4.43	7.36 \pm 0.02	8.37 \pm 0.83*
	DMSO	7.31	8.58	17.42

*: Presence of inhibition effect of essential oil on the reference strain DMSO: dimethyl sulfoxide; VO: vegetable oil

The use of the dilution method in agar medium allowed Nostro *et al.* (2004) to confirm the susceptibility of the MRSA and MSSA strains, including

ATCC 25923, to the essential oil of *Origanum vulgare* with thymol (24%) and carvacrol (14%). Their MIC was 0.125 after dilution in dimethyl sulfoxide (DMSO).

Results of in vitro test were in agreement with those of the authors mentioned above and confirm the antimicrobial efficacy of oregano oil against control strains. For further in vitro evaluation of the efficacy of topical solution or ointment containing essential oil, we suggest for the choice of the threshold value of the inhibition diameter to take into consideration the nature of the diluent, the diameter of the disc or the well and the dose to be impregnated on the disc or to be deposited in the well.

Microbiological quality in raw milk samples

The present trial revealed the occurrence of subclinical mastitis indicated by the CMT test without the presence at critical levels of major pathogens often investigated in quality control. These results are probably an expression of damage due to the minor germs that were not the subject of this control. These are mainly represented by coagulase-negative staphylococci (CNS) (e.g. *Staphylococcus chromogenes*) which cause an increase in SCCs but without effect on milk composition and production (Pyörälä and Taponen, 2009; Tomazi *et al.*, 2015). This finding was also cited by Bradley *et al.* (2007) in Great Britain and Wales who found the absence of major pathogens in 39% of samples representing subclinical mastitis and by Sampimon *et al.* (2009) in the Netherlands with an incidence of 20.8% in milk samples with a high somatic cell count greater than 250,000 cells/ml. In the present study, the somatic cell count was subjectively monitored by the CMT test which did not accurately reflect the status of cell immunity which could have been better controlled if an automated counter had been used, although in Algeria the cell count is not yet taken into account in the regulations as a criterion for the quality control of commercialised raw milk.

The microbiological analysis showed the presence of TMAB and coagulase-positive staphylococci in all the samples without their numbers reaching or exceeding the limits required by the Algerian state (Algerian Ministry of commerce, 2017). However, an absence of coliform, *Listeria monocytogenese*, and *Salmonella* in all samples, either at the beginning or the end of the trial was noted. Compared to Algerian standards (Algerian Ministry of commerce, 2017), the hygienic quality of all milk samples analysed was satisfactory regarding the limit criterion of major pathogenic germs counts independently of the sampling time, before or after the application of the essential oil.

Based on the overall results of the microbiological analysis (Table 3), a significant decrease ($P < 0.05$) in the number of TMAB after three days of essential oil application from values between 10^4 CFU/ml and 10^5 CFU/ml (90% of the samples) to values between 10^2 CFU/ml and 10^3 CFU/ml (70% of the samples) was observed. In agreement with the present finding, the supplementation in the concentrate mixture for healthy Holstein dairy cows with individual dose (2.5 and 5 g/head/day) of another oregano species (*O. majorana*),

mixed with clove and juniper in equal proportions as an essential oils blend improved the microbial profile of milk during the post-treatment period by a 20.7 and 17.9% reduction in total milk bacterial count (TMAB), respectively, compared to the control group (Al-Suwaiegh *et al.*, 2020).

For coagulase-positive staphylococci, there is a significant absence in 40% of the unit component samples ($P < 0.05$) after the application of the essential oil with the maintenance of a population counting from 1 CFU/ml to 50 CFU/ml in 60% of the samples before or after treatment ($P > 0.05$). Concerning the other bacteria (Coliforms, *Listeria* and *Salmonella*), a total absence of these bacteria was observed in all milk samples before and after the application of the essential oil. Therefore, the effect of this oil on them will not be retained for the rest of the discussion although other authors (Özkalp *et al.*, 2010) have observed in vitro an antibacterial activity of the oregano essential oil with different MICs against *E. coli* (250 µg/ml), *Listeria monocytogenes* (250 µg/ml) and *Salmonella enteritidis* (128 µg/ml).

Table 3. Prevalence of bacteria counts levels in raw milk samples before and after oregano essential oil applications

Bacteria	Counts levels (CFU/ml)	Prevalence (%) per unit component sample	
		Before	After
Total Mesophilic Aerobic Bacteria (TMAB) at 30 °C Limit*: 3×10^5 CFU/ml	$[10^2, 10^3 [$	0 ^a (0/50)	70 ^b (35/50)
	$[10^3, 10^4 [$	10 ^a (5/50)	30 ^b (15/50)
	$[10^4, 10^5 [$	90 ^a (45/50)	0 ^b (0/50)
	$> 10^5$	0 (0/50)	0 (0/50)
Coagulase-positive staphylococci (CPS) Limit*: 100 CFU/ml	Absence	20 ^a (10/50)	40 ^b (20/50)
	[1, 50]	62 ^a (31/50)	80 ^a (30/50)
]50, 80 [10 ^a (5/50)	0 ^b (0/50)
]80, 100 [8 ^a (4/50)	0 ^b (0/50)
	> 100	0 (0/50)	0 (0/50)
Coliformes thermotolerant Limit*: 5×10^2 CFU/ml	Absence	100 (50/50)	100 (50/50)
<i>Listeria monocytogenes</i> Limit*: 100 CFU/ml	Absence	100 (50/50)	100 (50/50)
<i>Salmonella</i> Limit*: Absence in 25 ml	Absence	100 (50/50)	100 (50/50)

Different superscript letters denotes a subset of TIME categories whose prevalence does differ significantly from each other at the 0.05 level using the z-test.

*: Algerian Ministry of Commerce (2017).

In vivo effect of oregano essential oil on counts of Total Mesophilic Aerobic Bacteria

Total aerobic mesophilic flora enumeration before and after the oily solution application shows that there is a most significant decrease ($P < 0.001$) in the number of bacteria in all samples. This finding is valid for both affected and healthy mammary quarters that showed counts below the limit value of 3×10^5 CFU/ml (Table 4). Comparison of the average count of the TMAB shows that there is a significant decrease ($P < 0.01$) from 13920.00 CFU/ml to 649.94 CFU/ml for healthy group and from 17040.00 to 1780.00 CFU/ml for affected quarters ($P < 0.05$) at the end of the trial (Table 4). These changes confirm the significant effect ($P < 0.01$) of EO on the overall mean TMAB number of all samples. This important antibacterial activity has been tested in vitro by the diffusion disc method on six standard strains of Gram-positive and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae*). These strains showed a degree of sensitivity quite similar to the essential oils of *O. vulgare* with MIC values of 31.25 $\mu\text{g/ml}$ to 125.00 $\mu\text{g/ml}$ (Sari *et al.*, 2006). Recently, the antimicrobial activity of *Origanum vulgare* was reviewed by Alagawany *et al.* (2020). It was attributed to the particular richness of its oil in phenolic compounds. Among several compounds, carvacrol, citral, thymol and trans-cinnamaldehyde were effective at low MIC against the majority of mastitis-causing bacteria (Gupta *et al.*, 2020). Carvacrol is the major compound of the oil used in our trial. It was known by the US Food and Drug Administration (FDA) as a safe flavouring substance when used in or on food to preserve it (European Commission, 1999). This compound and thymol were the two phenolic compounds often present as major compounds in oregano oil particularly that collected in our study area as described above. Thus, the bactericidal activity of carvacrol has been observed in vitro on several bacteria (*Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Lactobacillus plantarum*, and *Bacillus subtilis*) with a MIC varying according to the bacteria, from 5 mg per Petri dish for *Escherichia coli* and *Staphylococcus aureus* to 20 mg for *Lactobacillus plantarum*. This activity has been linked to the hydrophobicity and chemical structure of this compound, whose presence of a free hydroxyl group and a delocalised system allows the exchange of protons (Ben Arfa *et al.*, 2006).

In vivo effect of oregano essential oil on coagulase-positive staphylococci counts

The counting of coagulase-positive staphylococci showed values below the permitted limits which decreased after the application of the essential oil, regardless of the status of the quarters, healthy or affected. A statistically significant decrease was only found for one unit in the sample ($p = 0.02$). A non-significant effect ($P > 0.05$) of OEO at the end of the trial on the mean number of staphylococci in the samples from healthy and affected quarters was recorded (Table 4) despite an overall decrease from 34.80 CFU/ml to 18.80 CFU/ml and 34.00 CFU/ml to 20.80 CFU/ml for healthy and affected quarters respectively.

The efficiency of the EO of *Origanum vulgare* with carvacrol (92%) has been observed in Brazil (Pozzo *et al.*, 2011) on coagulase-positive strains of *Staphylococcus spp* isolated from bovine mastitis regardless of their resistance profile to antibiotics (tetracycline, penicillin, erythromycin, ceftiofur, ampicillin, cephalothin and oxacillin) used in the treatment of mastitis with MICs lower than those obtained with the use of the majority pure compound carvacrol (99.5%). Its antibacterial activity was demonstrated *in vitro* in the United States on *Staphylococcus aureus* isolated from clinical bovine mastitis with a MIC of 0.5% and a minimum bactericidal concentration (MBC) of 1.2% (Baskaran *et al.*, 2009). At a concentration of 98% , carvacrol had strong antimicrobial activity on *Staphylococcus aureus* including MRSA strains isolated from tank cow's milk with a MIC of 0.058 - 0.234 mg/ml evaluated by microdilution method and inhibition zones ranging from 19 to 45 mm (Keyvan and Tutun, 2019). This susceptibility of MRSA strains to carvacrol explains that registered by the *in vitro* control test in our study.

Table 4. Effect of oregano essential oil on mean counts of total mesophilic bacteria, staphylococci and their ratio

Variables	Quarter Status	Time	Mean	SD	Time fixed effects	
					F	Sig.
TMAB MEAN (CFU/ml)	Healthy	Before	13 920.00*	4 520.18	95.14	0.00
		After	694.00*	320.44		
	Affected	Before	17 040.00*	4 879.34		
		After	1 780.00*	1 463.56		
CPS MEAN (CFU/ml)	Healthy	Before	34.80*	23.69	2.48	0.13
		After	18.80*	19.01		
	Affected	Before	34.00*	29.02		
		After	20.80*	19.42		
RATIO CPS/TMAB	Healthy	Before	0.003	0.002	3.23	0.09
		After	0.057	0.093		
	Affected	Before	0.002	0.001		
		After	0.022	0.031		

*: Significant difference at $P < 0.01$ between the mean of variable and the lower microbiological limit (m) value using t test.

m= the threshold limit number (CFU/ml or CFU/g), below which the product is considered of satisfactory quality.

TMAB: total mesophilic aerobic bacteria; CPS: coagulase-positive staphylococci

Effect of oregano essential oil on the ratio of CPS count per TMAB

No significant change in the proportion of staphylococci to TMAB was noted (Table 4). However, an increase in ratios interpreted as an increase in the proportion of staphylococci relative to TMAB in both healthy and affected groups was recorded at the end of experimentation. The lack of significance of the effect of this oil on the mean overall ratio ($P > 0.05$) suggests that there was no significant change in the balance of the milk flora between the beginning and end of the trial (Table 4). This finding seems to be explained by the presence of multiple factors determining the SCP number and their proportion in the milk flora. Surveys on 27 dairy farms in France (Michel *et al.*, 2001) have shown that the level of microbial populations and the proportion between the flora of technological interest and spoilage flora are influenced by the hygienic practices of milking equipment and udder hygiene before and after milking. These measures had little influence on CPS which are very often present in non-negligible numbers either in milk samples with a low level of aerobic mesophilic flora (1.2×10^3 CFU/ml) where more than 50% of the milk contains at least 100 CFU/ml, or in milk with a higher level of mesophilic flora (1.5×10^4 CFU/ml) which have 37% of the samples at less than 100 CFU/ml. It has been suggested that the proportion of staphylococci is influenced by several factors such as the health status of the herd and, in particular, the presence or absence of cows with recurrent mastitis (Michel *et al.*, 2001).

CONCLUSIONS

The present in vivo study, reinforced by an in vitro control test, demonstrated an antibacterial effect of the topical oily solution containing essential oil of *Origanum vulgare* with carvacrol as a prevalent compound on the indicators of the microbiological hygienic quality of milk (TMAB) and mastitis-causing bacteria (CPS). It has also shown the ability of this oil to preserve the proportional balance of the bacterial population in milk. We suggest for further studies to take into consideration the emergence of minor germs and the recurrence potential of mammary infections due to antibiotic-resistant germs. Such research could help to assess the benefit-risk ratio of aromatherapy in the dairy sector and contribute to One Health.

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DYNAMICS OF ACIDITY (pH) AND TEMPERATURE DURING THE PRODUCTION OF TRADITIONAL MACEDONIAN GALICHKI KASHKAVAL

SUMMARY

The original technology and specificity of the raw milk are the most important characteristics for traditional cheeses. The present study aimed to investigate the acidity and temperature in the production processes in the first two days of the production of the traditional Galichki kashkaval during the summer period in a cheese plant in Galichnik village. The average chemical composition of sheep milk was examined. Acid development during renneting, syneresis, pressing, cheddarization, stretching and molding was investigated. The average active acidity of raw sheep milk after renneting was $\text{pH } 6.54 \pm 0.084$ and coagulation temperature of $37.96 \pm 0.104^\circ\text{C}$ and after curd pressure average pH was 6.23 ± 0.014 , while the temperature was $25.18 \pm 0.177^\circ\text{C}$. After cheddarization the pH of the ripened curd was in range of 5.45 ± 0.013 and the temperature of $19.08 \pm 0.149^\circ\text{C}$, after curd cutting pH was 5.32 ± 0.006 and a temperature of $19.08 \pm 0.058^\circ\text{C}$ and before stretching in hot brine was 5.28 ± 0.005 with a temperature of $19.77 \pm 0.117^\circ\text{C}$. These acid and temperature development enable proper plasticization of the cheese curd. The obtained values for the microclimate parameters in the traditional kashkaval ripening storage, the relative humidity of $77.21 \pm 0.159\%$ and the ambient temperature of $15.95 \pm 0.033^\circ\text{C}$ showed a statistically significant influence on the active acidity of the product. Only lactose showed a statistically significant influence on the active acidity and temperature of the intermediate products in the different stages of the production of the traditional kashkaval.

Keywords: active acidity, temperature, sheep milk, traditional kashkaval

INTRODUCTION

Development of acidity through the production process is an important parameter in cheese production. Acidity regulates the level and degree of

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syneresis, the degree of solubility of minerals during the production process, as well as the composition and pH of the final product. The concentration of hydrogen ions (pH) is a critical factor in several stages of cheese production and ripening.

Renneting parameters in cheese made of sheep milk are affected by physico-chemical properties, including pH, larger casein micelle, more calcium per casein weight, and other mineral contents in milk, which cause differences in coagulation time, coagulation rate, curd firmness, and amount of rennet needed (Park, 2007).

Each type of cheese has a characteristic pH which is an indication of the conversion of lactose to lactic acid in the process of cheese production (Lawrence, 1993). Acidity also prevents the growth of pathogenic bacteria, affects the activity of the coagulant during production and ripening, stimulates syneresis and affects the activity of enzymes during maturation. This affects the texture and taste of the cheese. Coagulation temperature and concentration of rennet are one of the factors which greatly influence the rennet-induced coagulation of milk during cheese formation.

One of the traditional cheeses with long history which is produced on mountain Bistra in the Republic of North Macedonia is kashkaval from the region in Galichnik. The specific climatic conditions, hilly mountain as well as the sheep breeding tradition, are excellent preconditions for the production of the cheese in this region (Srbinovska and Santa, 2017). Galichki kashkaval is produced traditionally from raw sheep's milk without the addition of starter cultures (Santa and Srbinovska, 2014). It belongs to the „pasta filata” cheeses which means “stretched curd”, that refers to a unique plasticization and stretching process in these types of cheeses.

There is a very small amount of available information on the pH and temperature dynamics during the production of Macedonian kashkaval. The purpose of this study was to examine the acidity (pH) and the temperature in the first two days of the production of traditional Galichki kashkaval during the summer period in a cheese plant near village Galichnik.

MATERIAL AND METHODS

Kashkaval in the cheese plant in Galichnik is produced during the summer, from the second half of June to the first half of August. The dairy plant has a daily capacity of 800 liters of milk for processing into kashkaval. For the production of Galichnik cheese, sheep's milk is used from its own farm, from morning and evening milking.

Dynamics of acidity and the temperature were controlled in different stages during production, as follows: raw milk reception; renneting; cutting and stirring the coagulum; before pressing the curd; after pressing the curd; during the curd ripening (chedaring); curd on pieces; before stretching the curd in hot brine; after kneading and molding, immediate after pulling the „navel” from the cheese and after removing the mold (next day).

The active acidity (pH-value) measurement was performed with a pH meter - Mettler Toledo. The temperature control of the product was performed with a calibrated alcoholic thermometer.

The following parameters were analyzed in raw milk: fat, protein, lactose and dry matter on Milk analyzer Lactoscan SA; active acidity pH - pH-meter Mettler Toledo; titration acidity - ° SH (titration Soxhlet-Henkel). The cheese composition was analyzed by standard methods: total solids by standard drying method at $102\pm 2^{\circ}\text{C}$ (IDF Standard 4A: 1982), fat (Gerber method), protein (Kjeldahl, IDF 20B:1993), salt, the contents of moisture on a fat free basis (MFFB) and fat in the dry matter (FDM) were calculated (Codex, 1978).

RESULTS AND DISCUSSION

Chemical parameters of sheep's milk used for the production of kashkaval

The average chemical composition of raw sheep milk that was used for the production of Galichnik kashkaval are shown in Table 1. The results are in accordance with the Rulebook on raw milk quality requirements (Official Gazette no. 96/2011, of the Republic of N. Macedonia).

Table 1. Chemical composition of raw sheep milk (%)

Parameter (n=90)	\bar{x}	STDV	$S_{\bar{x}}$	Min	Max
Fats	8.10	0.981333	0.126	5.81	9.72
Proteins	5.56	0.681768	0.088	3.79	6.41
Lactose	3.96	0.482478	0.062	2.71	4.56
Solids non fat	11.053	1.341408	0.173	7.57	12.71

Similar results regarding the milk fat content were obtained by Mioč *et al.* (2009), Gonzalo *et al.* (1994) and Pavić *et al.* (2002). Dozet *et al.* (1979) and Prpić *et al.* (2003) showed lower fat content in sheep milk $7.3 \pm 1.47\%$ and $7.81 \pm 0.84\%$. accordingly.

Mioč *et al.* (2009) found that the average protein content during lactation was $5.89 \pm 0.02\%$. According to the studies of Prpić *et al.* (2003), proteins in sheep milk in the same period were $5.59 \pm 0.54\%$ which are similar with our results. Pavić *et al.* (2002) found higher results which ranged from $5.94 \pm 0.05\%$ to $6.46 \pm 0.14\%$.

The average content of solids non fat in the milk was 11.05%, with variations from 7.6% to 12.71%. Antunac *et al.* (2007) found slightly lower values for the solids non fat of East Friesian sheep milk ($10.54 \pm 0.03\%$ to $11.00 \pm 0.03\%$).

In our studies, the lactose content showed a slightly lower content compared to the studies of other authors. Thus, in the studies of Antunac *et al.* (2007) the average value of lactose in sheep milk in the mainland of Croatia ranged from $4.89 \pm 0.01\%$ to $4.6 \pm 0.01\%$ for the same lactation period. According to Mioč *et al.* (2009), the average lactose content during lactation in the sheep breed *creska* was $4.39 \pm 0.01\%$.

Dynamic of pH acidity and temperature during the production process

The average active acidity of the examined raw sheep milk used for the production of Galichki kashkaval was 6.63 ± 0.083 in accordance with the Rulebook on requirements for the quality of raw milk ("Official Gazette" No. 96/2011, of the Republic of Macedonia), where the prescribed pH value for sheep milk is from 6.5 to 6.8. Similar results are reported by Pavić *et al.* (2002), Prpić *et al.*, (2003) and Park *et al.*, (2007) and Samelis *et al.*, (2019). The average temperature of the raw sheep milk in the examined period was 26.91 ± 0.261 °C. These values for the active acidity indicate a certain quality of the raw material due to the relatively good hygiene in the milking process of the sheep.

Table 2. Dynamic of pH acidity and temperature during the production process

No	Production phases	n	pH	t (°C)
			$(\bar{x} \pm S_{\bar{x}})$	
1	Raw milk	204	6.63 ± 0.083	26.91 ± 0.261
2	Renneting	139	6.54 ± 0.084	37.96 ± 0.104
3	Cutting and stirring the coagulum	128	6.47 ± 0.016	36.64 ± 0.235
4	Curd before pressing	100	6.49 ± 0.084	34.85 ± 0.078
5	Curd after pressing	415	6.23 ± 0.014	25.18 ± 0.177
6	Chedaring (baskija)	450	5.45 ± 0.013	19.08 ± 0.149
7	Baskija, slice cuted	560	5.32 ± 0.006	19.08 ± 0.058
8	Baskija before stretching in hot brine	542	5.28 ± 0.005	19.77 ± 0.117
9	Formed cheese in wheel	110	5.05 ± 0.014	52.45 ± 0.417
10	Cheese	105	5.36 ± 0.012	19.51 ± 0.506

The average coagulation temperature was 37.96 ± 0.104 °C. The amount of added rennet and the coagulation temperature influenced the development of acidity in sheep milk. Based on the analyses, it was found that in case of milk with low acidity (high pH value of 6.65), it is necessary to increase the amount of added rennet and the coagulation temperature needs to be higher (about 39.2 °C) to complete the coagulation in about 45 minutes. By shortening the renneting time, a slightly firmer curd can be obtained, and the whey can be separated faster. In the examined samples of the stirred coagulum, the average pH value was $6.47 \pm$

0.016, while the average temperature was 36.64 ± 0.235 ° C. Experiments have shown that curd with higher acidity (pH about 6.35) requires rapid mechanical processing of the grains to make the syneresis more intensive, while the curd with low acidity (pH – 6.6) has weak consistency. Curd acidification to a certain extent is of importance since it results in the characteristic fibre-like structure of the final cheese (Alichanidis and Polychroniadou, 2008). Delayed cutting of the coagulum, when it has reached a higher firmness, causes the faster separation of whey from the surface of the coagulum and reduces or completely stops the syneresis from the coagulum (Walstra, 2006).

The active acidity of the curd before pressing was measured at the time of processing the curd on the cheese desk. Based on the examinations, was concluded that in case when the processing of the curd takes a longer time, the curd can get cool, which slows down the acidification, the curd grains lose their stickiness, and thus later the process of pressing slows down. The mean active acidity was 6.49 ± 0.084 , with an average cheese dough temperature of 34.85 ± 0.078 ° C.

Dynamic of syneresis depends on the development of acidity, which determines the quality of processed curd. The results showed that the minimal pH of 5.46 and the temperature of 28.3 ° C in the curd after pressing occurs as a result of the low pH value of the milk intended for clotting, and in some cases due to improper processing of curd grains, resulting in improperly drained curd, with increased moisture and acidity.

The ripening of the curd (called Baskia) was at room temperature. The baskia then was slice cutted to separate the remaining whey and to obtain a uniform mass. In our study, the average pH of the cutted baskia was 5.32 ± 0.006 , with an average temperature of $19,08 \pm 0,058$, while the average active acidity of the baskia before stretching under hot brine was 5.28 ± 0.005 , with an average temperature of 19.77 ± 0.117 ° C, which means that lactic acid processes and changes in moisture content take place more evenly and allow the transformation of unplasticized curd into plasticized when immersed in hot water. The temperature of the hot brine was 73-75 ° C.

We have noticed that in case of lower active acidity (pH about 5.08 and temperature of 18.5 ° C) the curd plastication occurred and the curd became very crumble. On the other hand, in case at the maximum obtained pH value (about 5.48 and a temperature of 18.5 ° C), we noticed that the curd is too strong, processing in the hot brine is longer and generally does not stretch well. This is with accordance with Anifantakis (1991) who stated that the acidified curd is ready for further processing when its pH value is close to 5.2–5.3 for sheep's milk.

The average pH value of the cheese in the mold was 5.05 ± 0.014 , while the average temperature of the cheese was 52.45 ± 0.417 ° C. The obtained results are in accordance with the studies of Law and Tamime (2010) and according them if the stretching is performed too fast and the temperature of the brine is too low, the temperature of the stretched curd will be low. Therefore, it will be

difficult to perform the kneading and stretching of the curd, which will result to breaking and fat and moisture loss.

The average pH value of the wheel of cheese without rind on the first day of production was 5.36 ± 0.012 , with an average temperature of 19.51 ± 0.506 °C. The results in pH were lower in comparison with the results reported by Santa and Srbinovska (2014), where the pH on the first day of ripening the average pH of the Galichki kashkaval was 5.47 ± 0.07 . The pH values are in accordance with the results of Temizkan *et al.* (2014) on Kashar cheese made of ovine milk. Lower pH values were found in Kashkaval of Pindos cheese reported by Pappa *et al.* (2018).

Ripening temperature has a major influence on the rate of ripening and quality of cheese, and it is fairly characteristic of the variety (McSweeney *et al.*, 2017). The values of the ambient conditions in the room (relative humidity and room temperature) where the cheese was ripened in our research are shown in Table 3.

Table 3. Average values for the relative humidity and Temperature in the ripening room of the traditional kashkaval

	RH (%)	t (°C)
	$(\bar{x} \pm S_{\bar{x}})$	
Kashkaval ripening room	$77,21 \pm 0,159$	$15,95 \pm 0,033$

Table 4. Mean values for chemical composition of the final product – traditional Galichki kashkaval (%)

Parameter (n=3)	mean	SDEV	CV(%)
Total solids	60.813	0.833	1.370
Moisture	39.187	0.833	2.127
Fats	26.017	0.975	3.748
Protein	27.847	2.086	7.492
Fat in dry matter	42.772	1.022	2.389
Moisture on a fat free basis	52.963	0.437	0.825
Lactose	1.003	0.095	9.473
Salt	2.307	0.615	26.662

Chemical parameters of the Galichki kashkaval

After six months of production, the chemical composition of the final product was analysed. According to the data shown in Table 4, it can be seen that the dry matter content is $60.813 \pm 0.833\%$ and the moisture content is $39.187 \pm 0.833\%$.

Moisture on a fat free basis was $52.963 \pm 0.437\%$. Higher results regarding the fat content of the Galichki kashkaval were reported by Santa and Srinovska (2014). Similar fat and salt values to the present study but lower protein content was reported for 180-day Kashkaval of Pindos cheese (Pappa *et al.* 2020). According to the cheese classification of Codex Alimentarius, we can conclude that Galichki kashkaval belongs to the group of hard cheeses.

Interaction of pH and T on the chemical composition of milk and final product

According to the results from the multivariate regression statistical model, presented in Table 5, the production process of traditional Galichki kashkaval showed statistically significant influence at the level of $p < 0.001$ on the active acidity (pH-value) and the temperature of the intermediate products at different stages of the production of kashkaval.

Table 5. Interaction of pH and T on the chemical composition

Sources of variation	Degrees of freedom	pH ^a	T ^b
Model ^{a,b}	18	145.062,738***	9.894,192***
Product	10	2.434,865***	1.944,972***
Fat (F)	1	0,186 ^{NS}	1,589 ^{NS}
Lactose (L)	1	10,785**	9,063**
Protein (P)	1	0,604 ^{NS}	1,942 ^{NS}
Dry matter (DM)	1	1,801 ^{NS}	0,196 ^{NS}
Relative humidity in ripening room (F-RH)	1	38,604***	5,846*
Ambient room temperature (F_T)	1	42,420***	4,688*
F x P x L x DM	1	56,100***	0,069 ^{NS}
F-RH x M_T	1	38,808***	5,223*
Error	1.596	0,020	7.166
Total	1.614		
^aR² = 0,999; ^bR² = 0,991			

*** statistically significant at the level $p < 0,001$

** statistically significant at the level $p < 0,01$

* statistically significant at the level $p < 0,05$, ^{NS} statistically insignificant

From the chemical composition of raw milk, only lactose showed significant influence at the level of $p < 0.01$ on the active acidity and temperature of the intermediates at different stages of the production of traditional kashkaval.

The interaction of the components of raw milk (fat, protein, lactose and dry matter) showed a high significant influence at the level of $p < 0.01$ on the active

acidity of the intermediates at different stages of the production of traditional kashkaval. However, the chemical composition of raw milk did not show a statistically significant influence on the temperature of the intermediate products. The microclimatic conditions in the cheese ripening warehouse (relative humidity and air temperature) and their interaction showed a statistically significant influence at the level of $p < 0.001$ on the active acidity of the product, while they have a statistically significant influence at the level of $p < 0.05$ on the temperature of intermediates at different stages of the production of the kashkaval

CONCLUSIONS

Galichki kashkaval is the most famous traditional cheese in North Macedonia. In this study the acidity and temperature in all steps of the production process are presented. Based on the obtained results, there is a positive impact on the dynamics of active acidity and temperature on the product, as a result of the activity of microorganisms in lactic acid fermentation, which leads to a decrease in pH at certain stages in the production process. In the maturation phase, the pH gradually increases as a result of protein degradation.

According to the cheese classification of Codex Alimentarius, Galichki kashkaval belongs to the group of hard cheeses.

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INFLUENCE OF FOLIAR FERMENTED PLANT INFUSIONS ON ORGANICALLY GROWN BLACKBERRY

SUMMARY

The aim of this study was to examine the influence of nettle, dandelion and alfalfa infusions on the content of soluble dry matter and the dry matter yield of organically grown blackberry fruit. The fermentation period during the preparation of plant infusions was 14 and 21 days. First treatment with plant infusions was performed in mid-May, followed by treatments every 10 to 15 days. The treatments were conducted until the appearance of the first ripe blackberry fruits. The soil on which the blackberry plantation was established was analyzed, as well as the chemical composition of plant infusions, the content of soluble dry matter and the dry matter yield of blackberry fruit.

The soil was slightly alkaline. The contents of heavy metals lead and cadmium in soil were below the limit allowed for the soil intended for organic production. After 14 and 21 days of fermentation, the pH value and the content of nitrogen, phosphorus and potassium in the infusions was determined. The pH value of the nettle infusion was mildly acidic, while of dandelion and alfalfa infusions it was acidic. The highest content of soluble dry matter in ripe blackberry fruits was found in the treatment with dandelion infusion fermented for 21 days (9.51%), and the lowest in the treatment with 21 days fermented alfalfa infusion (8.80%). Statistically highly significant difference in the content of soluble dry matter was found in blackberry fruits treated with alfalfa infusions fermented for 14 days (F-14) and 21 days (F-21). The dry matter yields of blackberry fruits per unit area in treatment with F-14 nettle and dandelion infusions were higher than when treated with F-21 infusions. The opposite was obtained with the alfalfa infusion treatment, i.e., higher yield of fruit dry matter was achieved with F-21 treatment. The highest dry matter yield was obtained with the application of alfalfa F-21 infusion and amounted to 1 327.4 kg ha⁻¹. Statistically highly significant differences in the dry matter yield of blackberry

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fruits were found between the control treatment and treatments with the F-14 and F-21 alfalfa infusions.

Keywords: blackberry, organic production, plant infusions, dry matter yield

INTRODUCTION

Production of safe food without the use of pesticides, while preserving biodiversity and biological activity of the soil, is the basic goal of organic agriculture. With this method of food production, the application of mineral fertilizers must be eliminated from the production cycle. In such circumstances, in order to avoid a reduction in yield, there is a growing need for usage of organic fertilizers instead of mineral ones. Among organic fertilizers, foliar organic fertilizers, which can be used during the entire vegetation, are especially important. Care should be taken when using organic fertilizers, because their untimely and excessive use, due to intensive mineralization, can cause some negative consequences. Due to the negative consequences that can occur with the application of organic fertilizers, the use of foliar organic fertilizers is becoming more popular with organic food producers, because they simultaneously provide them with healthy products and enable the preservation of the environment.

By using infusions made from different plants, producers can expect a high-quality yield and good positioning on the market. At the same time, the use of organic foliar fertilizers does not have a negative impact on the natural environment. The use of organic foliar fertilizers essentially supports the concept of self-sustainable agriculture, in which fruit growing based on the principles of organic agriculture, has a significant place. It has been reported that after several treatments of plants with organic foliar fertilizers an increase in yield was observed, but also an improvement in the nutritional value of harvested fruits (Hoitink *et al.* 1997, Quarles, 2001). In a very short time, foliar fertilization can compensate for the lack of macro and micro elements that are not absorbed in sufficient quantities through the roots (Boshev *et al.* 2016). Foliar fertilizers are directly absorbed through the leaves and in limited quantities, unlike fertilizers that are absorbed through the soil (Fernandez and Eichert 2009).

A large number of plant species which can be used for preparation of quality foliar plant infusions grow freely in nature. Plant infusions, in addition to providing the plant with a sufficient amount of easily accessible nutrients, have partly insecticidal and fungicidal action due to the bioactive chemicals contained in them (Kim *et al.* 2005, Daoubi *et al.* 2005).

Having in mind the fact that plant infusions are environmentally friendly and do not pollute the environment, the undertaken research could make a significant contribution to the improvement of organic fruit growing in our country. Due to their biodegradability and eco toxicological acceptability, natural plant preparations are very popular with farmers in organic agriculture. This type of preparations has long been used on smaller farms in poor countries (Roy *et al.* 2005). Plant infusions can be a significant source of various trace elements for

plants, depending on the plant species and the quality of the soil on which these plants are grown (Popescu *et al.* 2010).

The production of berries according to the concept of organic food production, in the Republic of Srpska and Bosnia and Herzegovina is mainly focused for own needs and takes place on small areas. From the financial point of view, the economic sustainability of organic agriculture in B&H would be possible if there was a demand for the consumption of organic food or conditions for its export (Vaško and Kovačević 2020). The planned establishment of blackberry plantations based on the concept of organic production, was initiated in our country at the end of the 1990s with the help of donated funds. Currently, the largest blackberry plantation in B&H is located in Srebrenica, with a total area of 9.5 ha.

Blackberry fruits are an excellent laxative, they contribute to better digestion of food, improve blood count, regulate blood pressure and strengthen the body's resistance to arteriosclerosis and heart attack. Antioxidants found in blackberry fruits participate in preventing the harmful effects of free radicals, and thus occurrence of malignant diseases, they protect the cardiovascular system, slow down the aging of the body, and help fight the harmful effects of the sun on the skin. The nutritional value of blackberry fruit is reflected in the content of a large number of different compounds whose quantity depends on the variety, ecological conditions, applied cultivating practices, yield and degree of maturity at harvest (Nikolić, 2004).

It is estimated that cultivated blackberries are grown on more than 25 000 ha worldwide (Fernandez and Ballington, 2014). The yield of blackberry fruit in the conventional production of the varieties Thornfree, Thornes logan and Gente del Giardino (Giant) in favorable conditions ranges from 22 000 to 25 000 kg ha⁻¹, and in the variety Smoothstem up to 30 000 kg ha⁻¹, but on poorer soils yield falls below 15 000 kg ha⁻¹ (Stančević, 1990). The yields of blackberries grown in our agro-ecological conditions can reach up to 20 000 kg ha⁻¹ (Osmancevic and Brzica, 1991). The yield of blackberry cultivar Onyxa in the conventional method of production averaged 14 000 kg ha⁻¹ (Finn *et al.*, 2011). The obtained yield of blackberry fruit grown in organic production in 2012 was 7 400 kg/plant (Fernandez-Salvador *et al.*, 2015). Zavišić *et al.* reported that blackberry plants in organic production treated with nettle solution F-14 had the average yield of 10 550 kg ha⁻¹, and 10 260 kg ha⁻¹ when treated with F-21 nettle solution. The average yield of ripe fruit obtained from blackberry plants treated with F-14 dandelion solution was 10 210 kg ha⁻¹, and for those treated with F-21 solution was 10 370 kg ha⁻¹. The average yield of ripe blackberry fruit from plants treated with alfalfa solution F-14 was 10 190 kg ha⁻¹, and the application of F-21 treatment had yield of 10 970 kg ha⁻¹. An average yield of 9 015 kg ha⁻¹ of blackberry fruit was obtained in the control (Zavišić *et al.*, 2015).

The aim of this study was to examine the influence of infusions prepared with different plants and fermentation periods on the content of soluble dry matter and the dry matter yield of blackberry fruit grown in organic production.

MATERIAL AND METHODS

Experimental studies on the influence of nettle, dandelion and alfalfa infusions on the content of soluble and dry matter yield in blackberry fruit were performed in an organic blackberry plantation in the area of Kozarska Dubica, B&H. Field trials and laboratory testing lasted two years. For the treatment of blackberry plants, nettle, dandelion and alfalfa infusions were prepared with different fermentation periods, namely for 14 days and 21 days. Young plants of nettle, dandelion and alfalfa were used for the preparation of infusions. After harvesting, the plants were finely chopped and immersed in pots previously filled with rainwater. The nettle infusion was prepared by adding 1 kg of chopped young nettle plants to the pots with rainwater. For the preparation of dandelion and alfalfa infusions, 2 kg of young chopped plants were used. There were a total of 6 vessels, i.e. three for the preparation of solutions with a fermentation period of 14 days, and three with a fermentation period of 21 days. During the fermentation, the pots with chopped plants were kept in the shade and the contents of the pots were occasionally stirred. After the end of the fermentation cycles of 14 and 21 days, the plant infusions were filtered, and then diluted with distilled water in a ratio of 1:10.

Before the use of plant solutions, the control of their safety and chemical composition was performed. Microbiological control of plant infusions was performed at the Veterinary Institute "Vaso Butozan" in Banja Luka. The presence of following bacteria was examined in the extracts: *Salmonella*, *Staphylococcus*, *Clostridium*, *Proteus* species and *Escherichia coli*. Chemical analysis was performed in the laboratory of the Department of Agroecology at the Agricultural Institute of Republic of Srpska in Banja Luka.

The Čačanska bestrna variety was planted in an organic blackberry orchard. The distance between the blackberry bushes was 3 m x 1.5 m. The experimental trial on the blackberry plantation was set up according to a random block system in 4 replications. Each replicate consisted of five blackberry bushes.

During these tests, the first treatment with plant infusions was performed in mid-May, followed by treatments every 10 to 15 days until the appearance of the first ripe blackberry fruits. The treatments were performed with a Villager DM-25 motor sprayer.

The first harvest of ripe blackberry fruits was in the mid-July in the phase of physiological maturity. By the end of August, the blackberry fruits were selectively harvested on six occasions. Blackberry fruits were harvested from all zones of the bush; five fruits from each top, middle and lower part of the plant. The following properties were analyzed: chemical composition of plant infusions, content of soluble dry matter in blackberry fruit and dry matter yield of

blackberry fruit. Measurement of soluble dry matter content in blackberry fruit was performed with a manual refractometer MR200ATC, and the values were expressed as a percentage. The dry matter content was determined by drying 10.00 g of fresh blackberries in an oven at 105 °C up to a constant weight. Dry matter yield per unit area was calculated based on the blackberry fruit yield and dry matter content in blackberry fruits.

The obtained results were processed by the statistical method of analysis of variance (ANOVA), and the significance of the differences in the mean values was determined by the LSD test.

Agro ecological conditions: Soil samples for the chemical analysis were collected in a depth of 0-40 cm from the blackberry plantation grown in organic production before the experiment. Chemical analysis of the soil samples was performed in the laboratory for agrochemistry of the Faculty of Agriculture, Novi Sad. The weather conditions in the area where the experiment was performed were monitored during the course of research. For that purpose, the data of the Hydro-meteorological station at the Mlječanica site, which is located in the northwest of the Republika Srpska, B&H were used. The average amount of precipitation in the vegetation period was higher in relation to the perennial average (442.7 mm). The average air temperatures during the vegetation period (IV-VIII) were at the level of perennial average (18.9 °C), with slight deviations.

RESULTS AND DISCUSSION

The blackberry plantation was established on the brown earth soil, which is characterized by a favorable water-air regime. The results of chemical analysis of soil samples taken before the start of the research are shown in Table 1.

Table 1. Results of soil chemical analysis

Depth (cm)	pH in KCl	pH in H ₂ O	Humus (%)	P ₂ O ₅ mg/100g	K ₂ O mg/100g	Pb (mg kg ⁻¹)	Cd (mg kg ⁻¹)
0 – 40	6.95	7.67	3.21	9.72	20.68	15.5	1.78

The results of soil analyses showed that the blackberry plantations were established on the soil with pH value of 7.67 in water, while pH in KCl was 6.95. Based on the pH results, this soil is considered to be a soil with a slightly alkaline reaction. According to the humus content, which was 3.21%, this soil is considered to be moderately supplied. The content of easily accessible phosphorus at a depth of 0-40 cm was low, 9.72 mg 100 g⁻¹. The soil contained 20.38 mg 100 g⁻¹ of K₂O and was well provided with this biogenic element. The content of heavy metals and potentially toxic elements in agricultural soil, lead and cadmium, was below the limit allowed in the soil intended for organic production. Based on chemical analysis of the soil sample from a depth of 0-40 cm, it can be concluded that it was suitable for organic blackberry production.

Before the beginning of foliar application of plant infusions obtained from nettle, dandelion and alfalfa, analysis of their chemical composition and microbiological safety was performed. Microbiological control confirmed that plant infusions were safe for use in organic blackberry production. The results of chemical analysis of infusions after 14 and 21 days of fermentation are presented in Table 2.

Table 2. Chemical composition of plant infusions after 14 and 21 fermentation days

Plant infusion	No. of fermentation days	pH value	N (mg l ⁻¹)	P (mg l ⁻¹)	K (mg l ⁻¹)
Nettle	F-14	6.4	56.8	56.3	700.2
	F-21	6.5	53.9	55.4	695.9
Dandelion	F-14	5.2	41.0	42.5	1 205.3
	F-21	5.2	45.4	44.9	1 245.7
Alfalfa	F-14	5.4	105.6	40.2	670.4
	F-21	5.5	101.7	39.5	668.2

After 14 days of fermentation, the pH value of the nettle infusion was weakly acidic (6.4), while that of dandelion (5.2) and alfalfa (5.4) was acidic. After 21 days of fermentation, the pH value of the nettle infusion was slightly higher (6.5), but still weakly acidic. The pH value of the dandelion solution remained unchanged after 21 days of fermentation (5.2), while the value of the alfalfa infusion increased slightly, but the solution was still acidic. After 14 days of fermentation, the nettle infusion had a nitrogen content of 56.8 mg l⁻¹, phosphorus 56.3 mg l⁻¹ and potassium 700.2 mg l⁻¹. After 21 days of fermentation, a chemical analysis of the nettle solution revealed a slightly lower content of all examined macroelements, and the largest decrease was in the content of potassium (695.9 mg l⁻¹). The dandelion infusion obtained after 14 days of fermentation had a nitrogen content of 41.0 mg l⁻¹, phosphorus 42.5 mg l⁻¹ and potassium 1 005.3 mg l⁻¹. The content of potassium in the dandelion infusion was higher than in both nettle and alfalfa infusions. In the F-21 dandelion infusion, a slight increase in all tested macronutrients was found, and the largest increase was in the potassium content (1 245.7 mg l⁻¹). At the same time, in the F-21 alfalfa infusion, a lower content of nitrogen, phosphorus and potassium was determined compared to the content in the solution that was obtained after 14 days of fermentation. The largest decrease was in the nitrogen content (101.7 mg l⁻¹).

Blackberry fruit contains a large number of different organic and inorganic ingredients, which have nutritional, medicinal and protective value. The content of soluble dry matter in blackberry fruit can vary, depending on several factors (agro-ecological conditions, variety characteristics, degree of fruit maturity, yield, etc.). Farming practices affect the content of soluble dry matter which is higher in

production where chemical plant protection is used compared to the organic method of cultivation where pesticides are not used (Schneider *et al.* 2011). The content of mineral substances of certain elements (P, K and Mg) is higher in organic than in conventional production.

During this research, the content of soluble dry matter in ripe blackberry fruits that were harvested from plants treated with nettle, dandelion and alfalfa infusions was monitored, and the results are shown in Table 3.

Table 3. Dry matter content (%) in ripe blackberry fruit treated with different infusions

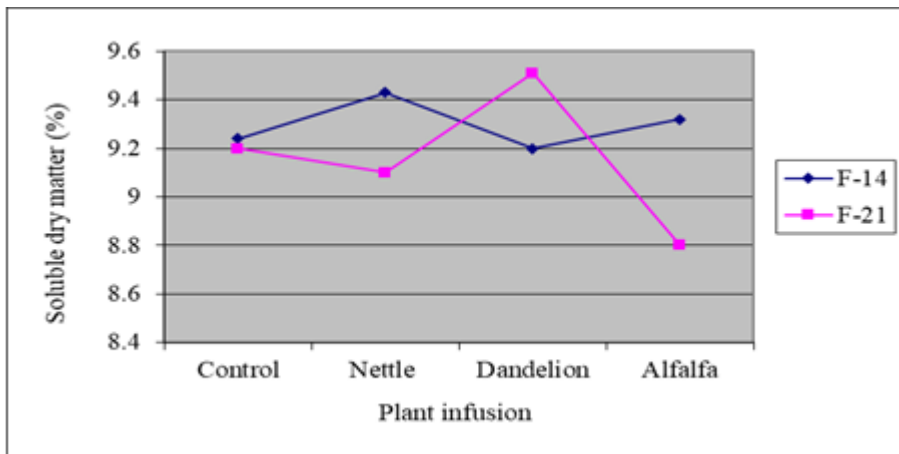
Fermentation period		Plant infusion			
		Control	Nettle	Dandelion	Alfalfa
F-14		9.24	9.43	9.20	9.32
F-21		9.20	9.07	9.51	8.80
\bar{X}		9.22	9.25	9.36	9.06
Level		A		B	AB
LSD	0.05	0.52 ^{ns}		0.37*	0.72 ^{ns}
	0.01	0.70 ^{ns}		0.50**	0.98 ^{ns}

A-plant infusion; B-fermentation period; AB-interaction; ns-not significant; *significant at $p < 0.05$; **very significant at $p < 0.01$

The average values of soluble dry matter content in ripe blackberry fruits harvested from the control variant F-14 was 9.24%, while in the control variant F-21 the determined value was slightly lower, 9.20%. The highest content of soluble dry matter in blackberry fruit was found after treatment with F-21 dandelion extract (9.51%), and the lowest after treatment with alfalfa extract of the same number of fermentation days (8.80%). No statistically significant differences in the content of soluble dry matter in ripe blackberry fruits were found between control and the applied plant infusion treatments. At the same time, statistically highly significant differences in the content of soluble dry matter in blackberry fruits harvested from shrubs treated with alfalfa infusions with different number of fermentation days were observed. Blackberry fruits treated with F-14 alfalfa infusion had a higher content of soluble dry matter (9.32%) than fruits harvested from shrubs treated with F-21 solution (8.80%). Treatments of blackberry fruits with plant solutions that had higher content of nitrogen and potassium resulted in higher content of soluble dry matter in fruits (Table 2). Changes in the content of soluble dry matter in blackberry fruits treated with plant infusions prepared with two fermentation periods are shown in Graph 1.

The content of soluble dry matter in ripe blackberry fruits can vary from 10 to 14% (Gavrilović and Janda 1977, Šoškić, 1984). According to the results reported by Nikolić (2004), ripe blackberry fruits contain 9-14% of soluble dry matter, while in wild blackberries the content of these substances varied from 6 to

13.1%. In addition to climatic conditions during the ripening period, the content of soluble dry matter in the fruit is significantly influenced by the cultivated variety. In the research of Milošević *et al.* (2012) cultivar Čačanska bestrna had the lowest content of soluble dry matter compared to other cultivars in the experiment. The content of soluble dry matter does not change significantly during storage in most blackberry varieties (Repajić *et al.* 2019). During a two-year experiment with organically grown blackberry varieties Black Diamond, Metolius, ORUS 1939-4, ORUS 2635-1, Obsidian and Onyx, the content of soluble dry matter in ripe fruits varied from 11.2% to 14.4 % (Fernandez-Salvador *et al.*, 2015). The content of soluble dry matter in wild blackberries grown in Croatia ranged from 9.4–11.5% (Purgar-Dujmović *et al.*, 2012). The yield of blackberry dry matter depends on a number of factors, and particularly assortment, cultivation system, applied farming practices and agro-ecological conditions. The dry matter yields obtained in our study are presented in Table 4.



Graph 1. Content of soluble dry matter in blackberry fruits treated with infusions of nettle, dandelion and alfalfa relative to control

The average dry matter yield of blackberry fruits per unit area was the lowest in the control. The obtained dry matter yields of blackberry fruits in the treatments with F-14 nettle and dandelion infusions were higher compared to the treatments with F-21. At the same time, in the treatment with alfalfa solution the opposite was found, i.e., higher yields of fruit dry matter were achieved with F-21 treatment. The differences in the dry matter yields of blackberry fruits between control and treatment with F-14 solution were statistically significant. There were also statistically significant differences between the control treatment and the treatments with F-21 dandelion and alfalfa infusions.

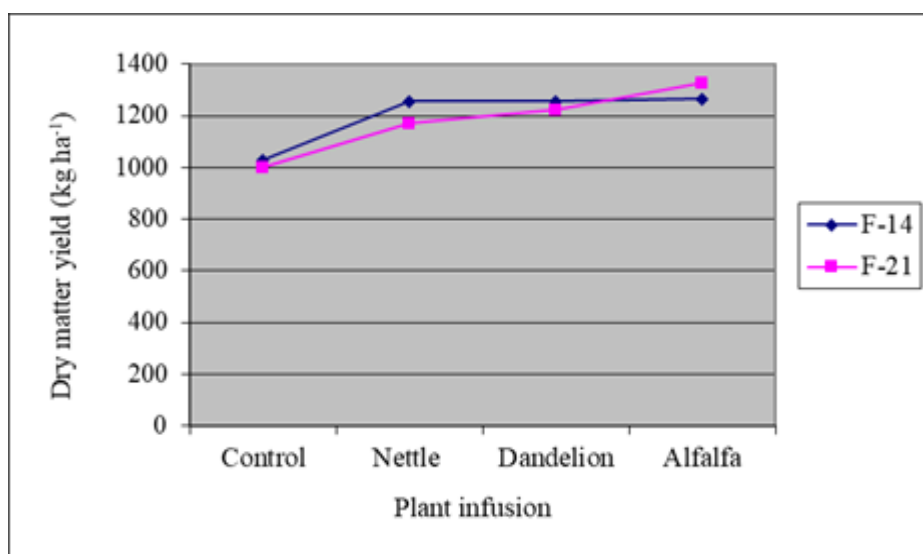
Statistically highly significant differences in the dry matter yield of blackberry fruits were found between the control treatment and the F-14 and F-21 alfalfa infusion treatments. The highest yield of dry matter of blackberry fruits

was achieved with the application of F-21 alfalfa solution (1 327.4 kg ha⁻¹). The differences in the dry matter yield of blackberry fruits between the applied treatments with infusions of different fermentation periods were not statistically significant. Changes in dry matter yield in blackberry fruits treated with the solutions from different plants with two fermentation periods are shown in Graph 2.

Table 4. Average yield of blackberry dry matter (kg ha⁻¹) treated with plant infusions

Fermentation period		Plant infusion			
		Control	Nettle	Dandelion	Alfalfa
F-14		1 028.0	1 254.9	1 255.4	1 265.3
F-21		1 001.0	1 169.6	1 223.4	1 327.4
\bar{X}		1 014.5	1 212.3	1 239.4	1 296.4
Level		A		B	AB
LSD	0.05	174.2*		123.2 ^{ns}	246.4 ^{ns}
	0.01	236.1**		166.9 ^{ns}	333.9 ^{ns}

A-plant infusion; B-fermentation period; AB-interaction; ns-not significant; *significant at $p < 0.05$; **very significant at $p < 0.01$



Graph 2. Dry matter yield in blackberry fruits treated with infusions of nettle, dandelion and alfalfa relative to control

CONCLUSIONS

Based on the chemical analysis of the soil sample from a depth of 0-40 cm, it can be concluded that it was a weakly alkaline and suitable for organic

blackberry production. The content of heavy metals and potentially toxic elements lead, and cadmium was below the limits allowed in the soil intended for organic production. Microbiological tests have shown that nettle, dandelion and alfalfa infusions are safe and can be used for foliar treatment of blackberry in organic production.

The type of plant and the length of the fermentation period influenced the pH value of the plant solution. After 14 and 21 days of fermentation, the pH value of the nettle infusion was slightly acidic, while dandelion and alfalfa solutions were acidic. The tested plant infusions can be used in organic production as a good source of N, P and K. F-14 and F-21 alfalfa infusions had the highest content of nitrogen (105.6 mg l^{-1} and 101.7 mg l^{-1} respectively), while the F-14 and F-21 dandelion infusions had the highest potassium content (1205.3 mg l^{-1} and 1245.7 mg l^{-1} , respectively). Fermentation period did not significantly affect the content of elements in the individual plant infusions.

The highest content of soluble dry matter in ripe blackberry fruits was obtained after treatment with F-21 dandelion extract (9.51%). The highest yield of dry matter of blackberry fruits was achieved with the application of F-21 alfalfa infusion ($1327.4 \text{ kg ha}^{-1}$).

The use of plant infusions in organic blackberry production increased the average dry matter yield.

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CHARACTERIZATION AND GEOCHRONOLOGY OF THE DELTAIC SYSTEM FROM JEQUITINHONHA RIVER, BRAZIL

SUMMARY

Deltaic systems possess singular morphology and represent a portion of the Brazilian large fluvial systems, with complex dynamics and mouths located at the Atlantic Ocean. Landscapes generated are occupied by diverse human activities over the coast and depend on a certain degree of dynamic equilibrium to its maintenance. The mouth of Jequitinhonha River, located at the extreme South of Bahia state, has suffered great transformations over the last decades, causing coastal erosion processes due to fluvial discharge reduction, thus changing the local dynamic. So, to understand the preterit deltaic dynamic and the possible agents of the current changes, we proposed an approach utilizing grain size and Optically Stimulated Luminescence (OSL) of the deposits by the river mouth, with the processes currently demonstrated. The main results point to mixed depositional environment over the sampling points and additionally in depths, demonstrating cyclicity of depositional agents and energy. Depositional ages obtained were 2.72 ± 0.23 (Ky) for the left riverbank, 1.1 ± 0.15 (Ky) for the right riverbank, and the youngest deposit located at the front island, with absolute dating of 0.555 ± 0.065 (Ky). The results evidence a tendency of coastal progradation during the last thousands of years, prior to anthropic interventions which diminished and regulated fluvial discharge of Jequitinhonha River, resulting in advancement of local coastal erosive processes.

Key words: Deltaic system, Grain size, Coastal erosion, Holocene.

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INTRODUCTION

It is becoming acknowledged that water is likely to be the most pressing environmental concern (Aaron *et al.*, 1999). The flow of rivers is part of a greater flow, the planet's water cycle, which sustains not only the flow of water but the entire web of life (Karr & Chu, 2020). River networks, the backbone of most landscapes on Earth, collect and transport water, sediment, organic matter, and nutrients from upland mountain regions to the other rivers, lakes, seas and oceans (Spalevic *et al.*, 2013.; Willett *et al.*, 2014.; Nikolic *et al.*, 2019.; Chalise *et al.*, 2019.; Spalevic *et al.*, 2020.; Santana *et al.*, 2021.; Tavares *et al.*, 2021).

Large rivers from Brazil are known to develop highly complex deltaic systems over their river mouths in the Atlantic Ocean, due to higher sedimentary load, sediment rework capability by marine processes tend to be lower (Christofoletti, 1981; Suguio, 2003; Rossetti, 2008). Generally, these deltaic systems locally enlarge the coastal plain, causing prominent coast progradation, preserving Quaternary environmental conditions in the Brazilian coast, as described by Martin *et al.* (1993), thus raising their importance in the paleo environmental context.

According to Goudie (2006) the terminology delta is used from 450 years B.C., cited by Herodotus to describe sandy deposits in triangular shapes located at the mouth of Nile River, resembling the delta letter from Greek alphabet. However, more specific studies within the theme generated important conceptual changes, becoming more generic, increasing the scope related to the term, until reaching complex deltaic systems, comprising, according to Scoot and Ficher (1969), Wright (1978) sedimentary accumulation from fluvial deposits in coastal environments, comprising sub-aqueous, sub-aeolian, and in adjacent or closely related, including secondarily reworked deposits by diverse marine agents; waves, currents, and tides.

Observing Brazilian coastal context, Martin *et al.* (1993) concluded that a delta encompasses several forms of coastal accumulation in a broad spectrum, containing beach deposits, dunes, tidal flats, swamps, mangroves, lagoons, barrier island, bays, in addition to channel deposits and river mouth. The concept of deltaic systems is used to denominate geomorphological units present in progradation zones, connected to the fluvial system, built originally from sediments carried by rivers that flow towards a permanent body of water in a stillness state. Therefore, the speed of fluvial currents decreases from the contact with the new environment, so the sediments are gradually/selectively deposited, continuously, depending on its mass and morphology, and marine processes posteriorly could rework such material.

The progradation of the coastal line occurs as the contribution from fluvial sediments achieve higher energy over the capability of costal processes to erode them, thus, the sedimentary balance is highlighted in the landscape, generating deltaic patterns accordingly with the interaction between coastal/fluvial and marine processes. In contrast, once there is a sedimentary deficit, the ocean advances over the continent due to decline of material from the river discharge, generating new debris, thus resulting in coastal erosion.

Deltas, due to natural resources availability, especially hydrocarbons, demanded various researches in energy resources, as for its economic relevance,

these studies focus on prospecting, exploration of mineral coal and petroleum (Suguio, 2003). The knowledge regarding deltaic sedimentation is combined to the deltaic cycle, constituted by constructive and destructive phases. The constructive phase is formed by an active period of intense sediment progradation confined to the distributors in the river mouth, resulting in an abandonment of these distributors, making an extensive sediment progradation. Posteriorly, the destructive phase encompasses deposit rework by active processes in the receptor basin, presenting typical marine features (Coleman and Gagliano, 1965; Scruton, 1960; Suguio, 2003).

From this panorama, the aim of this work is to perceive the preterit and current dynamic of Jequitinhonha River deltaic system, to understand the environment framework through grain size analysis and Optically Stimulated Luminescence (OSL) geochronology of depositional events in the deltaic system, correlating those with local and regional studies. Thus, presenting aggradation tendencies from the Holocene dynamics, in comparison to the current coastal erosive context established in the region, pointing to possible natural and anthropic factors as agents of changes in the deltaic system.

MATERIAL AND METHODS

Study area. Jequitinhonha River source is located at the Espinhaço ridge, South of Diamantina city, in the state of Minas Gerais, in approximately 1.260 m high, and its mouth located on the Atlantic Ocean in the municipality of Belmonte, extreme South of Bahia state, Brazil (Figure 1).

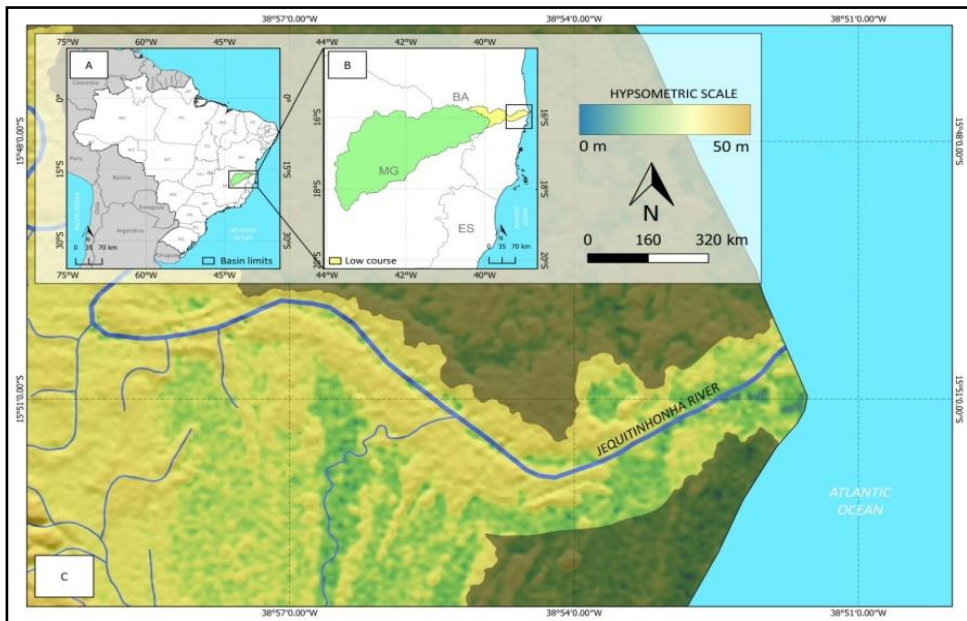


Figure 1. Location of the study area: A) position in Brazil. B) Jequitinhonha River basin and the study area in the low course. C) Study area with the hypsometric scale. The geographic coordinate system is UTM Sirgas 2000. Datum: WGS 84. Source: (ANA, 2016).

Its drainage basin comprehends a total area of approximately 70.315 km², and the portion located in Bahia state covers approximately 3.996 km² (SEI, 2011). The largest portion of Jequitinhonha River basin is located at the Northeast part of Minas Gerais state, with a size of 66.319 km², equivalent to 93.6% of its total area, while the smallest area is located at the extreme South of Bahia state, equivalent to 6,4% of its total area. The Jequitinhonha River basin limits are the following: Pardo River drainage basin to the North; Santo Antônio River drainage basin to the South; São Francisco River drainage basin to the West, trespassing Bahia state limit, entering Minas Gerais state; Atlantic Ocean to the East, (SEI, 2004).

The lithostratigraphic units from Jequitinhonha River are highly heterogeneous, encompassing diverse geological Groups and Formations, due to its large territorial extension. However, as this research focuses in the coastal plain, specifically the deltaic system, Barreiras Group is considered the base lithology to structure the coastal plateau relief in the Southern region of Bahia, representing the coastal plain inner boundary with distinct distances to the ocean. According to Araújo *et al.* (2006) Barreiras Group consists in the most expressive geological unit from Brazil, occurring from the North of Rio de Janeiro state to the state of Amapá, along with part of Brazilian coast, presenting sediments with variable grain size from Miocene.

Past Barreiras Group consolidation in Bahia Southern region, the Group was eroded and reworked by marine and fluvial processes during the Quaternary, constituting part of the available sediments along the coastal plain, arriving at the present geomorphological aspects, according to evolutive model proposed by Arai (2006) in a series of papers about the theme. In mapping performed by CPRM (2006) five (5) sedimentary deposits are highlighted at Southern Bahia coastal plain, these deposits possess diverse origins, related to marine transgressive and regressive moments, alluvial debts, lagoon deposits, and Barreiras Group rework establishing the core of the deltaic environment.

According to the map of land use and land cover elaborated by Silva (2012) the native vegetation consists of restingas over the coastal plain and diverse Atlantic Forest phytophysognomies over the coastal plateau and valley bottom. The region has been historically used by production of cocoa and livestock, however over the last decades; forestry occupies large areas, replacing older crops, due to pulp industry installation in the region, demanding such feedstock. Another important land use in the region is Itapebi hydroelectric power station, located in the low course of Jequitinhonha River, causing negative effects to channel flow regularization and dynamics, discussed by Silva (2012), elaborating important parameter to the discussions of this work.

Nascimento *et al.* (2007) correlates geomorphological continental processes with marine processes to explain the predominant direction South-North from the longshore drift present in the extreme South of Bahia, inferring that higher intensity is related to the large input of solid particulate material carried by the large Jequitinhonha and Pardo Rivers, originating the coastal plain

largest portion over the last thousands of years, thus creating the complex deltaic system analyzed. Lastly, another relevant characteristic observed at the study area consists in the connectivity in between the diverse drainage basins in the region, mainly among Jequitinhonha and Pardo Rivers, possessing secondary channels connecting both basins, generating certain complexity degree in their delimitation. Drainage catchments with current features are commonly found in plain and low areas over the coastal plain, where the same channel distributes water to two distinct sub-basins, exhibiting wide influence as tide ebb and flow, floods, and droughts.

Methods. Before methodological procedures, there is an importance to highlight marine terraces and delta from Jequitinhonha River as component of landscape surficial covers, described by Perez Filho and Rubira (2019). These elements are potential geomorphological indicators of environmental changes, directly associated to variations in the deltaic dynamics, thus surficial covers are the discussed and dated object within the analyzed system.

The OSL technique was chosen to obtain absolute dating data from the material composing surficial covers, due to its analytic characteristics encompassing the main hypothesis from surficial covers genesis. Therefore, during the material transport, diagnostic materials (Feldspar and Quartz), were exposed to solar radiation during enough time to reset stored luminescence and posteriorly deposited, starting new environment radiation absorption as described by Sallun *et al* (2007).

The sampling for OSL dating was carried out with all the necessary care to avoid sample direct contact with sunlight, as recommended by the laboratory responsible for processing the samples. To collect a sample, a 60 cm length and 6 cm diameter PVC tube (Polyvinyl chloride) was used, properly packaged after removed from the deposit until taken to laboratory for analysis. The sampling depths vary between 70 and 80 cm, contemplating homogenous material with apparent sandy texture.

At the laboratory Datação LTDA, the OSL samples were analyzed utilizing the SAR – Single Aliquot Regenerative protocol, described by Wintle and Murray (2000, 2006) where 15 repetition aliquots were carried out for each sample. Alongside OSL sampling, grain size samples were collected in different depths to identify texture changes within the deposit. Posterior to the sampling procedure, the samples were sent to the soil laboratory of Agriculture Faculty (FEAGRI – UNICAMP), where grain size analysis was performed, sorting five fractions of sand, clay, and silt by sieving and pipette methods, described by Camargo *et al.* (1986) and EMBRAPA (1997).

After measuring particle size, a statistic analysis was performed. The free source software Sysgran 3.0 was used (Camargo, 2006). Among the data generated by the software, statistical parameters were average, median, selection, asymmetry, and kurtosis with method develop by Folk & Ward (1957). Cumulative frequency graphics of the fraction in each sampling point were generated utilizing the same software.

RESULTS AND DISCUSSION

We have selected 3 collection points in the study area to apply the described methodology, located at the left and right riverbank and the front island of Jequitinhonha River, according to figure 2 found by the river mouth, and these points represent the current deltaic system dynamics.



Figure 2 – Sampling points location by the mouth of Jequitinhonha River. LB – Left Riverbank; RB – Right Riverbank; FI – Front Island. Source: (ANA, 2016, IBGE, 2015).

Grain size analysis performed to characterize the surficial covers present at the study area represent the dynamism of this deltaic system located at the river mouth, for the analyzed fraction vary significantly along the selected points as per depths. Grain size cumulative frequency curves (figure 3) enlighten the difference between samples, as the cumulative patterns diverge, evidencing influence of distinct genetic agents in the construction of sedimentary deposits.

At the right riverbank it was possible to notice higher presence of thinner fractions as depth increases, this point also presented higher variation of gran size fractions in higher depths, indicating multiple transport agents and variable energy during the deposit genesis, possibly connected to fluvial discharge oscillation within the area. The left riverbank presented a distinct behaviour, pointing to distinct deposition dynamics over the study area.

Thicker fractions had a bigger presence on surficial layers in contrast to deeper ones on the right riverbank deposit, clearly indicating a higher energy dynamic over the genesis of youngest layer. Whilst the left riverbank presented lower variation in the parameters analyzed with closer cumulative frequency

curves and a lack of muddy material. Therefore, higher concentration of thicker sand fractions in left riverbank, indicate more energetic environment and/or influence of marine deposits over this deposit.

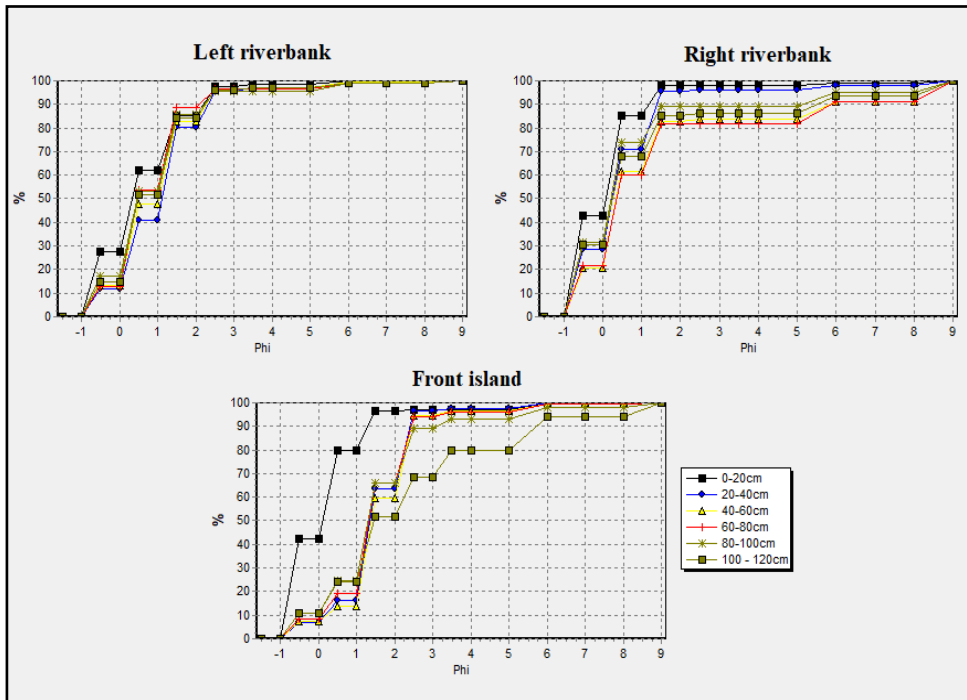


Figure 3 – Grain size cumulative frequency curves in Phi scale. (Sysgran 3.0.)

We have performed grain size analysis following the statistic and descriptive method from Folk and Ward (1957) which is presented in table 1. These parameters emphasize observations previously made, demonstrating heterogeneity within the deposits. Regarding mean classification, modal fraction varied between very coarse, coarse, and medium sand, material commonly deposited in fluvio-marine environments.

According to Martins (2003), material selection measured by grain size analysis could be an indicative of the agent involved in transporting the sedimentary material, related to beach deposits moderate selection, comparatively to poorly selected related to fluvial transport. Thus, we observed processes intercalation, both in depths and sampled points, indicating non prevalence of a unique process in each of the points.

Asymmetry parameter is another important factor to observe in table 1, for positive asymmetry is generally associated to beach deposits, whereas negative asymmetry is linked to fluvial deposits, complementing the selection parameter analysis according to Martins (2003). We observed that not all analyzed deposits achieve the described combination, possibly due to influence of a mixed environment, where presented parameters are crossed, thus complicating a clear interpretation.

Table 1. Grain size statistic parameters by Folk and Ward (1975)

Left riverbank								
Depth cm	Class.	Median	Selection		Asymmetry		Kurtosis	
0-20	Coarse sand	0,3266	1,048	Poorly selected	0,1532	Positive	0,7435	Platykurtic
20-40	Medium sand	1,115	1,01	Poorly selected	- 0,0909	Approx. symmetric	1,11	Mesokurtic
40-60	Medium sand	1,029	0,999	Moderately selected	- 0,0550	Approx. symmetric	1,097	Mesokurtic
60-80	Coarse sand	0,4535	0,834	Moderately selected	0,3087	Very positive	1,134	Leptokurtic
80-100	Coarse sand	0,4567	1,005	Poorly selected	0,11	Positive	1,106	Mesokurtic
100-120	Coarse sand	0,4783	0,868	Moderately selected	0,2898	Positive	1,102	Mesokurtic

Front island								
Depth	Class.	Median	Selection		Asymmetry		Kurtosis	
0-20	Very coarse sand	0,0835	0,676	Moderately selected	-0,1324	Negative	0,877	Platykurtic
20-40	Coarse sand	0,2524	0,860	Moderately selected	0,02516	Approx. symmetric	0,597	Very platykurtic
40-60	Medium sand	0,3564	2,83	Very poorly selected	0,6965	Very positive	3,024	Extremely leptokurtic
60-80	Medium sand	0,3669	2,875	Very poorly selected	0,6954	Very positive	2,951	Very leptokurtic
80-100	Coarse sand	0,2185	1,567	Moderately selected	0,3701	Very positive	1,723	Very leptokurtic

Right riverbank								
Depth	Class.	Median	Selection		Asymmetry		Kurtosis	
0-20	Coarse sand	0,1011	0,845 5	Moderately selected	0,0922	Approx. symmetric	0,8612	Platykurtic
20-40	Medium sand	1,356	0,931 4	Moderately selected	-0,1203	Negative	1,184	Leptokurtic
40-60	Medium sand	1,393	0,898 9	Moderately selected	0,1712	Positive	1,402	Leptokurtic
60-80	Medium sand	1,329	1,092	Poorly selected	- 0,01297	Approx. symmetric	1,468	Leptokurtic
80-100	Medium sand	1,307	1,483	Poorly selected	0,1525	Positive	2,121	Very leptokurtic
100-120	Fine sand	1,466	2,621	Very poorly selected	0,4984	Very positive	1,607	Very leptokurtic

Source: Sysgran 3.0.

OSL dating analysis, table 2, provided data to identify distinct depositional events in relation to surficial covers genesis on the banks of Jequitinhonha River and front island. Therefore, the left riverbank was identified as the eldest with 2.72 ± 0.23 (Ky), followed by the right riverbank with 1.1 ± 0.15 (Ky), and the most recent deposits found within the front island, presenting absolute dating of 0.555 ± 0.065 (Ky). The data indicate an heterogenous dynamic in relation to the sampling points, corroborating data obtained from grain size analysis, pointing to distinct depositional events, suggesting channel preferential migration to the right over the last 1000 years B.P., furthermore, the front island presented most recent depositional events, relative to the last 500 years B.P. Similar depositional ages were found by Rocha *et al.* (2019), by the Paraíba do Sul River, demonstrating correlation between the processes analyzed.

Table 2. Parameters used to measure OSL dating.

Sampling point	Coordinates	Height (m)	Depth (cm)	^{232}Th (Ppm)	$^{238}\text{U} + ^{235}\text{U}$ (Ppm)	^{40}K (%)	Dose Rate ($\mu\text{Gy}/\text{year}$)	Aliquots	P (Gy)	Years (Ka)
Left riverbank	15°50'39"S 38°52'13"W	3	0,8	19,070 $\pm 0,687$	6,731 $\pm 0,04$	0,493 $\pm 0,07$	3.675 ± 135	15	10,0	2.72 \pm 0.23
Right riverbank	15°51'10"S 38°51'50"W	4	0,8	12,110 $\pm 0,436$	3,325 \pm 0,27	0,943 \pm 0,13	2.760 \pm 245	15	3,0	1.1 \pm 0.15
Front island	15°50'45"S 38°51'50"W	6	0,7	18,490 $\pm 0,666$	5,256 \pm 0,20	0,955 \pm 0,13	3.600 \pm 245	15	2,0	0.555 \pm 0.065

Source: Original.

Fluvial discharge and mean sea level oscillations during the Holocene and specially during the Late Holocene is an asset to explain the depositional ages from deposits close to the river mouth obtained through OSL analysis, as they are directly related to the construction and enlargement of the local coastal plain cyclically in different moments. Classic models representing mean sea level oscillations over the Brazilian and South American vast coastline consists in important point for the discussion, although not concordant between one another, the models present general tendencies of the processes investigated in this work, given that these are local variables when considering the dimension and diversity of the Brazilian coast.

So, models elaborated by Suguio *et al.* (1985), Tomazelli (1990), Coen *et al.* (2005), Rull *et al.* (1999), Martin *et al.* (2003), Angulo *et al.* (2006), Alves and Rossetti (2017) present the main fluctuations of mean sea level over the last 8 thousand yeas B.P., displaying clear regressive tendency in all periods evaluated, with presence of short-term transgressions over the last 5 thousand years B.P. Therefore, the natural tendency presented is the progradation during great part of the Holocene, particularly during the Late Holocene, period corresponding to the absolute dating obtained.

Discussing marine influence over fluvial systems and the formation of paleo-mangroves in the Southern coast of Pernambuco and Southern region of Bahia (Fontes *et al.* 2017, Cohen *et al.* 2019, Lorente *et al.* 2020, Martins *et al.* 2020), describe relevant climate events occurred during the Middle and Late Holocene, pointing to possible raise in mean sea level during 7,400 and 5,350 years A.P., due to presence of palynologic records in intra-continent estuarine systems, currently located until 34km from the actual coastline.

Climatic conditions of lower humidity could be the agent responsible to generate marine influence advancement peak (5,350 years A.P.), thus confirming classic models. Fontes *et al.* (2017) points to a continuous and regressive tendency of mean sea level, strongly influenced by an increment in fluvial discharge from Jucuruçu River in Southern Bahia, caused by higher humidity available during the last thousands of years. Therefore, this tendency is probably suggested to Jequitinhonha River during the same period.

Contrary to the natural tendencies previously described, in the last decades, we observed an inversion in the dynamics presented in Jequitinhonha River mouth, which is suffering severe fluvial and marine erosion as observed in fieldwork, figure 4, performed in the study area.



Figure 4 - Houses and commercial establishments new the mouth of Jequitinhonha River under risk due to marine erosion advancement.

Once processes occurring in the coastline of Bahia state were described by Dominguez *et al.* (2012), there was a highlight to mean sea level rise in the last decades, important variable in this context, however it should be avoided to understand erosion processes in the Brazilian coast, as the variable confuses the comprehension of particular and regional events. Therefore, the main processes related to the dynamics of river mouths that might cause erosion are channel lateral migration in case of small water courses, shifts in mouth bar configuration and natural oscillations of solid and liquid flow. Cases of severe erosion could be associated to anthropic interventions as the construction of ports and sediment bars in river, thus unbalancing the deltaic system.

Observations made by Dominguez *et al.* (2012) are corroborated by Silva (2012) when performing temporal analysis of orbital and non-orbital historical

series of images prior to and post Itapebi hydro electrical power plant installation in the low course of Jequitinhonha River, demonstrating the changes observed in the channel layout and in the front island, which decreases in size over the last decades. According to Silva (2012), channel flow regularization caused by the hydroelectric enterprise brought important upstream modifications, promoting channel enlargement, which currently decreased energy, and in the front of river mouth, suffers from the advancement of marine processes.

Strength loss in fluvial discharge has been modifying the entire coastline dynamic, which is also under an erosion moment, particularly on Jequitinhonha River right bank. Regarding the river channel, there is a notable increase in sandbanks and fluvial islands, formed due to the current low transport energy, creating difficulties in boats navigability, and reducing human traditional activities, directly impacting communities residing in this location.

CONCLUSIONS

Evidences from the past and present are important assets to comprehend the diverse variables that might influence the dynamics of complex deltaic systems and might aid in future impacts mitigation facing the current scenario of global environmental changes. Therefore, through methodology applied, it was possible to identify a natural progradation tendency, which intensified during Late Holocene and nowadays is under transformation at the mouth of Jequitinhonha River, considering that marine processes are causing coastline erosion, thus harming several human activities established at the location.

OSL dating demonstrate that all surficial covers close to the mouth of Jequitinhonha River was formed in the last 2000 years B.P., elaborated by several cycles of greater and lesser energy in the deltaic system, with mixed fluvio-marine contribution, inferred by grain size analysis, with the front island as the most recent in relation to the riverbanks, which also present an evidenced temporal lapse between one another, indicating preferential migration to the right. These results point to cyclicity within the deltaic system in natural condition, with progradation tendency.

Dynamic equilibrium observed during Late Holocene was disturbed by fluvial discharge reduction and regularization due to hydro electrical enterprise installation upstream, occasioning new hydrodynamic conditions to the system, where marine processes are superimposing fluvial processes, thus eroding the current coastline. In case this new dynamic is maintained, the deltaic system could even disappear, originating a new estuarine system, implying in large local changes, which might reverberate regionally, transforming the geosystemic dynamic in several hierarchic levels.

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THE EFFECT OF DIFFERENT NANO-, BIO- AND CONVENTIONAL MINERAL FERTILIZERS APPLICATION ON SOME MORPHOLOGICAL CHARACTERISTICS OF MAIZE

SUMMARY

Effective management of nutrient application is important part of the crop production puzzle and it seems that nano-fertilizers may have high potential for achieving sustainable crop production. A field experiment was carried out to investigate the effect of adding different nano-size and biological fertilizers on maize growth under various irrigation regimes. The experiment conducted under optimal irrigation level (up to ~50% field capacity) which is applied from the beginning of the reproductive period. Fertilizer's treatments included control (Nf; no-fertilizer application), N biofertilizer (Bio-N), P biofertilizer (Bio-P), nano-chelated B (Nano-B), nano-chelated Zn (Nano-Zn), complete nano-fertilizer (Nano-C) and conventional mineral NPK fertilizer. Bio-P was the best treatment in terms of grain yield, ear length, biological yield, number of the kernels per row, length of ear leaf and straw yield traits, while Nano-Zn was the best treatment for increase of protein content and Nf was the best treatment for increase of oil content. Bio-N was the best treatment in terms of leaf area, ear diameter and hundred grain weight, while Nano-B was the best treatment for plant height, harvest index, stem diameter, number of the row per ear and number of the kernels per ears traits. Nano-C and NPK are not outstanding for any of the traits. Nano-Zn had good effect on high yield and high protein content while nano-B was good for better performance of plant height, stem diameter, number of the row per ear, harvest index and number of the kernels per ears traits. Such an outcome could be used in the future to advise good recommendation strategies for recommendations for maize and other crops in other areas of the world.

Keywords: Grain yield, Micronutrient, Nano-chelated fertilizers, Yield components

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INTRODUCTION

Mediterranean semi-arid areas are characterized by low erratic rainfall and water shortage is the most severe restriction for crop production. Although cereal production is increased through applying the modern agricultural technologies, the arid and semi-arid areas have not greatly benefited from this increase. Also, it appears that plant production in drier agro-ecological zones, such as Mediterranean-type climate areas of Middle East is usually constrained by water limitation as well as nutrient limitation (Ryan *et al.*, 2012). Available soil moisture has a significant influence on nutrient use efficiency, and it determines crop yield. In arid and semi-arid areas, the loss of organic matter and low fertility are of great concern which causes low biomass production and high rates of organic matter decomposition. Also, the main challenge in the future decades will be the task of meeting food needs with less water, especially in arid and semi-arid regions and a watering strategy that whereby water supply is reduced below maximum levels is allowed with minimal effects on yield performance (Geerts and Raes 2009).

Nutrient deficiencies have been reported for a long period of time as a result of decline in soil organic carbon status in semi-arid regions (Sahrawat and Wani 2013). During the last decades, application of the fertilizers has been increased in arid and semi-arid areas. Nutrient management is a fundamental concern from the economic perspective and there are a range of options available for this purpose (Golzarfar *et al.*, 2012). The utilization of bio-fertilizers has become important in agriculture for their potential role in improving soil fertility and crop production (Bhardwaj *et al.*, 2014). Bio-fertilizers containing strains of symbiotic and non-symbiotic microorganisms which are beneficial bacterial or fungal inoculants that improve uptake of nutrients by crop roots.

Nitrogen (N) and phosphorus (P) are the two essential nutrients for maize growth and development; therefore, their bio-fertilizers could strongly improve the plant performance (Janmohammadi *et al.*, 2012). It has been recognized that conventional mineral fertilizer may cause some problems such as polluting water basins, destroying micro-organisms and friendly insects; however, it seems that bio-fertilizers application can overcome these problems (Chen, 2006). Most farmers of arid and semi-arid regions apply only N and P fertilizers that supply macronutrients, while micronutrients shortage is prominent (Ryan *et al.*, 2012). Although much lower levels of micronutrients are needed for obtaining satisfy yield and quality, they play vital role in plants development (Marschner, 2012). Up to now, the main source of supplying the micronutrients are mineral bulk fertilizers, while recently the tendency to use new fertilizers in nano-size (Nano-fertilizers) is increased (De Rosa *et al.*, 2010).

Nano-fertilizers are innovative agricultural input which is aimed to release nutrients into the soil gradually, avoiding environmental damages (Sekhon, 2014). In nano-fertilizers, nutrients can be encapsulated by nanomaterials, coated with a thin protective film, or delivered as emulsions or nano-particles (De Rosa *et al.*, 2010). Nano-fertilizers could be able to release nutritional elements in a

controlled manner as a reaction to different environmental fluctuations, so that it can enhance plant growth effectively more than conventional fertilizers (Naderi and Danesh-Shahraki 2013). Nano-fertilizers provide the nano-scale or nano-structured nutrients in a controlled release and lead to an increased efficiency of the nutrients, improve nutrient use efficiency and decrease costs of environmental protection (Sekhon, 2014).

Despite the plenty of information available on application of some nanoparticles on crops, there was not sufficient information about efficiency of nano-fertilizers under water scarcity condition. Therefore, the present investigation was carried out to evaluate the impact of some bio-fertilizers and nano-fertilizers under deficit irrigation on yield and yield components of maize in Mediterranean-type environment.

MATERIAL AND METHODS

Field experiments were carried out at the Moghan (46° 46'E and 39°36'N), Iran, during the growing season of 2014. It has warm and humid summers and temperate winters with dry winds and short freezing period and average annual rainfall was about 335 mm. The soil type was a clay loam, pH 7.22 and EC =2.35 dS.m⁻¹, organic matter 0.85%, potassium 306.4 mgkg⁻¹, phosphorous 15.8 mgkg⁻¹. The mean temperature and total rainfall during the growth season was 21°C and 124 mm, respectively. The trial was conducted in a randomized complete block designs with three replicates. The water deficit irrigation was 50% soil water content in field capacity level during initiation of reproductive growth until maturity stage. At mentioned area, the clay loamy soil has sufficient depth (more than 1 meter) and field capacity was at 33% and wilting point at 16% by volume for the surface to 100-cm soil layer.

Seeds of single cross 704 hybrid were hand sown on 27 April in 5 cm depth of soil. Each experimental plot area was 25 m² (5×5m) with 0.65 m spacing between rows and 0.2 m spacing between plants. Soil was tilled by moldboard ploughs during the August 2013 and seedbed preparation was carried out by disc plough, disks, leveler and furrower during April 2014. Fertility treatments consisted control (no-fertilizer), nitrogen bio-fertilizer (contains *Azotobacter vinelandii* strain O4), phosphorous bio-fertilizer (contains phosphate-solubilizing bacteria; *Pantoea agglomerans* strain P5 and *Pseudomonas putida* strain P13), nano-chelated boron, nano-chelated zinc, complete nano-fertilizer and conventional mineral NPK (nitrogen, phosphorus and potassium). Bio-fertilizer was applied as seed inoculation just before planting. Conventional mineral NPK consisted of 180 kg N ha⁻¹ in the form of urea, 100 kg P ha⁻¹ in the form of super phosphate, and 50 kg K ha⁻¹ in the form of potassium sulphate. Half of the N and all of P and K were applied before sowing (incorporated by disk). The remaining N was applied as a top dressing one month after sowing.

Nano-chelate fertilizers were obtained from the Fanavar Sepehr Parmis Company, Iran and due to the calcareous nature of the region soil micronutrients nano-fertilizers were applied as foliar spray at three times (2000 ppm) including 9-

leaf stage, stem elongation and heading. Nano-fertilizers contained nanoparticles of zinc oxide, boric acid or combination of absorbable forms of key micronutrients elements (Fe, Cu, Zn, B, Mn). Two hand weeding were carried out at 20 and 40 days after sowing date, respectively. Relative water content (RWC) was measured in leaves adjusted to ear at the beginning of grain development stage (BBCH-scale=71) according to Barrs and Weatherley (1962). Chlorophyll content was measured on ten leaves of a plant at each plot, using a SPAD-meter at the beginning of seed development stage. The central two rows from each plot were harvested at maturity and biological yield, straw yield, seed yield and harvest index were measured. Different agronomic traits including plant height, length of ear leaf, hundred kernel weight, kernel number, number of the kernel per row, number of the row per ear, cob diameter, ear diameter, ear length, stem diameter, leaf area per plant, biological yield. Content of protein and oil in grain (or kernel) were measured using a Near-infrared seed analyzer (Zeltex).

Treatment by Trait (TT) analysis was used to data analysis and plots were generated by the GGE biplot software (Yan, 2001; <http://www.ggebiplot.com>). This statistical method has been described in detail by Yan and Tinker (2005) and Yan *et al.* (2007).

RESULTS

The TT biplot of mean performance of coconut treatments explained 59% (41 and 18% for the first and second principal components, respectively) of the total variation of the standardized data (Fig. 1).

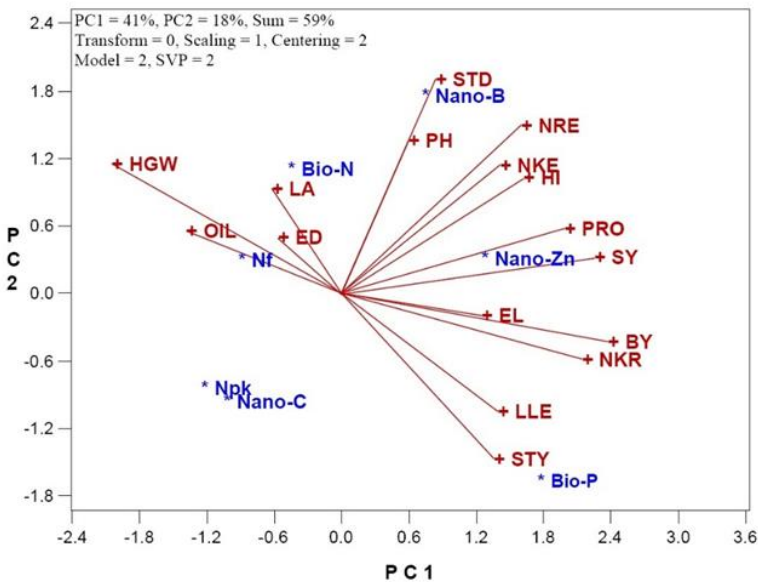


Fig. 1. Vector view of treatment by trait (TT) biplot indicating the interrelationship among traits under different fertilizer treatments (Nano and Bulk). For traits abbreviations, refer to the text.

The traits were considered as the tester and the treatments as entries. This relatively moderate percentage variation reflects the accuracy of interrelationships among the measured traits across different treatments. In the TT biplot, a vector is drawn from the biplot origin to each marker of the traits to facilitate visualization of the relationships between and among the traits. Provided that the biplot described an enough amount of the total variation, the association coefficient between any two traits is approximated by the cosine of the angle between their vectors (Yan and Rajcan 2002). On this premise, two traits are positively correlated if the angle between their vectors is an acute angle ($< 90^\circ$) while they are negatively correlated if their vectors are an obtuse angle ($> 90^\circ$). LLE and STY, PRO and SY, STD and PH, OIL and HGW were positively associated (an acute angle) as shown in Fig. 1. These relationships suggest that it is possible to combine higher seed yield with higher protein content in a single genotype. Also, these traits were positively correlated with each other (acute angles); NRE and NKE with HI; EL and BY with NKR (Fig. 1). STD and PH traits were not correlated with OIL and HGW due to near right angles and similar association was observed for LLE and STY traits with NRE, NKE and HI (Fig. 1). LLE and STY had negative correlation with OIL and HGW traits (Fig. 1).

Fig. 2 is biplot showing the polygon view of the treatment \times trait analysis on the morphological traits based on first two principal component axes. The treatment(s) at each vertex (vertex treatment) of the polygon in the biplot were the best in terms of the trait(s) found within the sector demarcated by any two lines that meet at the origin of the polygon.

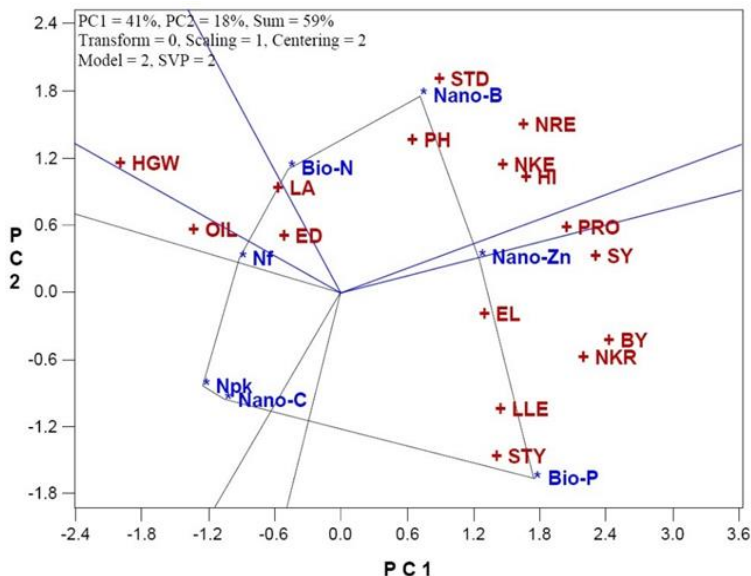


Fig. 2. Polygon view of treatment by trait (TT) biplot showing which fertilizer treatment (Nano and Bulk) expressed to the highest values for which traits. For traits abbreviations, refer to the text.

From Fig. 2, Bio-P was the best treatment in terms of seed yield, EL, BY, NKR, LLE and STY indicating that it can be used as the best fertilizer in the corn production. Even though Bio-P was identified for good performance in these traits, it was not the best for other remained traits, indicating that these traits might not be a good trait-indicator for seed yield. Nano-Zn was the best treatment for high protein content while Nf was the best treatment for high oil content. From Fig. 2, Bio-N was the best treatment in terms of LA, ED and HGW indicating that it can be used as the best fertilizer for achieving good performance of these traits while Nano-B was the best treatment for PH, HI, STD, NRE and NKE traits. Nano-C and Npk were also vertex treatments but no trait was found in their respective sector, an indication that they are not outstanding for any of the traits (Fig. 2). In other word, none of measured traits could not perform in high magnitudes regarding Nano-C and Npk treatments.

In the context of treatment-by-trait analysis, an ideal treatment (Nano-B) has been defined as the treatment that combines several good traits in its performance. An ideal treatment should possess the highest mean performance across traits (i.e., longest projection onto the average tester coordinate (ATC abscissa) axis and shortest entry-vector, thus, it should be close to the ideal treatment represented by the innermost concentric circle with an arrow pointing to it (Yan and Rajcan 2002). Such ideal treatment can, therefore, be used as a reference check in subsequent trials where the set of traits will be measured. In the biplot displayed in Fig. 3, the single-arrow line that passes through the biplot origin is referred to as the ATC abscissa, and on this line is ranked the treatments in terms of their performance.

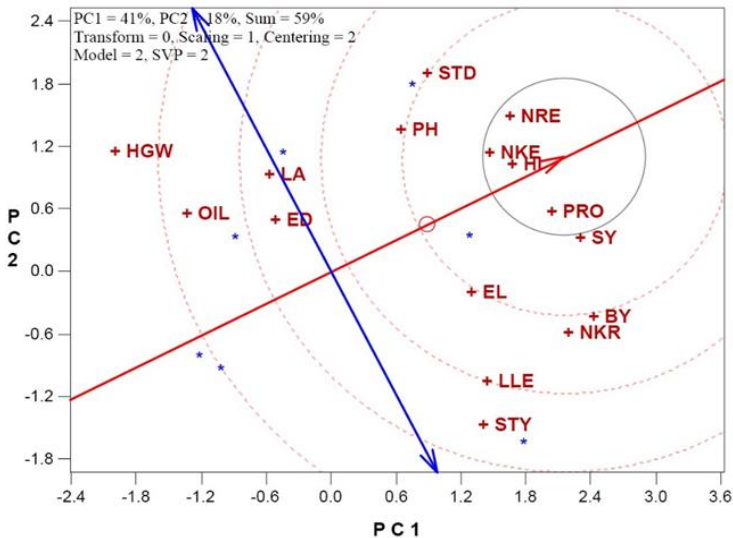


Fig. 3. Ideal test view of treatment by trait (TT) biplot, showing the relationships of different fertilizer treatments (Nano and Bulk) with ideal entry. For traits abbreviations, refer to the text.

The double-arrow line (ATC) divides the ATC abscissa into two at the middle (YAN *et al.* 2007). The portion of the ATC towards the right displays the above average treatments and towards the left shows those treatments below average. Based on this biplot, the treatments that performed above average were Nano-B, Nano-Zn, Bio-N and Bio-P treatments; while Nano-C, Npk and Nf treatments performed below average in terms of measured traits (Fig. 3). Nano-B is closest to the position of an ideal treatment and it is ranked the highest in term of morphological performance because it is desirable in terms of most of the traits. This treatment could serve as a good fertilizer for corn production.

A vector is drawn from the biplot origin to each marker of the treatment to enhance visualization of the relationships between and among the treatments (Fig. 4). The vector length of a treatment measures its effect (positive or negative) with others (Yan and Tinker 2005). The cosine of the angle between the vectors of any two treatments approximates the correlation coefficient (degree of association) between the treatments. From Fig. 4, Npk and Nano-C positively correlated, and it shows they all gave similar performance in the measured traits. Npk and Nano-C with Nano-Zn, and Bio-P with Bio-N were negatively correlated and indicated different performance. Nano-B did not show any association with Bio-P as well as Npk and Nano-C treatments (Fig. 4).

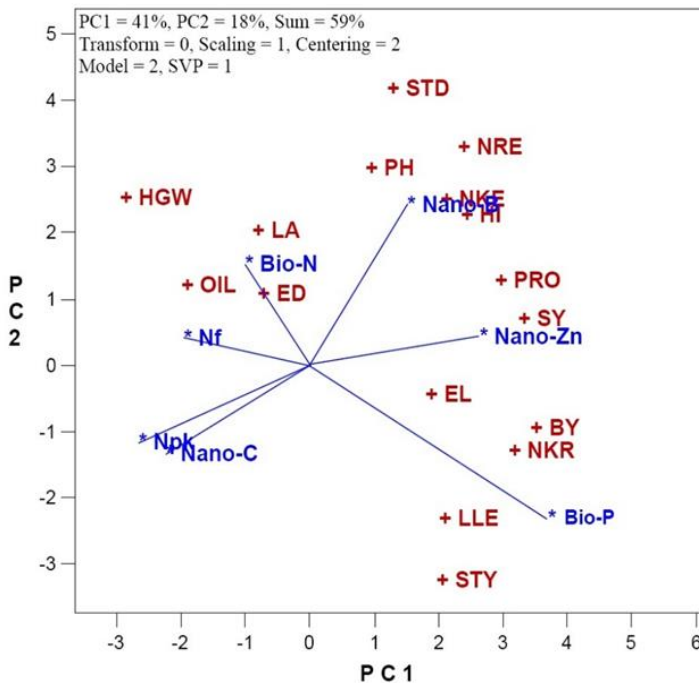


Fig. 4. Ideal entry view of treatment by trait (TT) biplot, showing the relationships of different fertilizer treatments (Nano and Bulk) with ideal entry. For traits abbreviations, refer to the text.

Ranking of various fertilizer treatments for seed yield (SY) performance indicated that Nano-Zn was the best treatment regarding high seed yield following to Nano-B and Bio-P fertilizer treatments, but the other treatments could not produce good yield performance in decreasing order Bio-N > Nf > Nano-Zn > Npk (Fig. 5). Zinc is the essential element in function of some enzymes and its application may efficient the ability of maintaining high yield. Using nano-size zinc had positive effects on obtaining high yield performance.

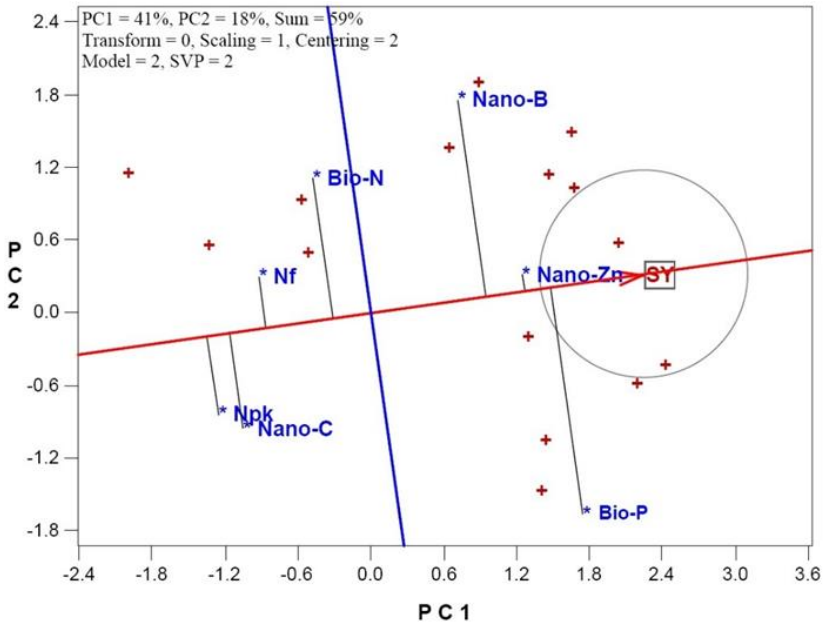


Fig. 5. Vector view of treatment by trait (TT) biplot, showing the relationships of different fertilizer treatments (Nano and Bulk) with target trait (SY, seed yield). For traits abbreviations, refer to the text.

DISCUSSION

Nano-fertilizers or bio-fertilizers play an important role when they are compared to conventional mineral fertilizers. Nanotechnology in many fields is in its primary stage, seeing new innovations it tells that it has a great scope and for any technology to that object there will be interested. We found that nano-Zn had good effect on increase of yield and protein content in comparison to the other fertilizer treatments. Zinc has general deficit in the world and its amount intake through daily food is very low, thus by application of zinc fertilizer there are least chances of indirect supply to human (Rameshaiah *et al.*, 2015). The nano-size zinc can be used to get a diffused and soluble zinc, and equal ratios between surface area and size of nano-particles should be carefully designed (Malik *et al.*, 2014). Also, for better performance of plant height, stem diameter, number of the row per ear, harvest index and number of the kernels per ears traits, nano-B could be used but nano-C could not affect the measured traits.

Nano-fertilizers are capable to hold bountiful of nutrient ions due to their high surface area and release it slowly and steadily that commensurate with crop demand. Subramanian *et al.* (2008) reported that nano-fertilizers can be used to control the release of nutrients from the fertilizer granules. They improve the nutrient use efficiency while preventing the nutrient ions from either getting fixed or lost in the environment and have high use efficiency and can be delivered in a timely manner to a rhizospheric target. It is still unclear whether type of nanotechnology for use in agriculture will has any long-term impacts on human or the environment; thus, further investigation into the impact that the nano-nanotechnology may has been studied (Rai *et al.*, 2012). It is difficult to predict the impact of nanotechnology on fertilizers' application in the future, for researchers, this insight may allow them to contribute nano-fertilizers in field.

For obtaining high kernel yield, these traits: ear length, number of the row per ear, number of the kernels per ears, biological yield, harvest index and protein content would be effective and useful. Several authors have attempted to determine relation between the characters on which the selection for high yield can be made. Annapurna *et al.* (1998) found that seed yield was correlated with plant height, ear diameter, number of seed per row and number of rows per ear. Knowledge about the traits' association is a great importance for success in selections to be conducted in breeding programs. The effects of different traits on seed yield were studied in 90 genotypes by Geetha and Jayaraman (2000) and they reported that number of seeds per row exerted a direct effect on yield.

Grain yield is the complex trait in maize and any change in any component leads to the yield loss whereas it is very sensitive to any crops. In this study we emphasized to determine the correlation coefficient of the traits via vector-view of biplot in order to understand and identify the correlated traits that play an important role in selection and breeding for improvement proposes. Therefore, these traits must be analyzed to know their relationship and their contribution on grain yield and we found ear length, number of the row per ear, number of the kernels per ears, biological yield, harvest index and protein content were the most effective traits on yield. It is clear that the biplot method is an excellent tool for visual data analysis because compared with conventional methods, the biplot approach has some advantages (Yan *et al.*, 2007). The first advantage of the biplot is its graphical presentation of the data, which enhances ability to understand the patterns. The second is that it is more interpretative and facilitates pair-wise treatment or trait comparisons. The third advantage of biplot method is that it facilitates identification of possible positive or negative interactions of treatment versus traits.

CONCLUSIONS

Based on the results which we get in this research conclusions are: (i) maize grain yield was positively associated with ear length, number of the row per ear, number of the kernels per ears, biological yield, harvest index and protein content traits, (ii) nano-B was the best fertilizer treatment while nano-Zn produced high yield and high protein content and (iii) no-fertilizer (Nf),

conventional NPK and nano-B were not good fertilizers for most of the measured maize traits.

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ALLELIC STATE OF WX-GENES AND THEIR RELATIONSHIP WITH GRANULOMETRIC STRUCTURE OF GRAIN STARCH IN COLLECTIVE SAMPLES OF WINTER TRITICALE

SUMMARY

The study was conducted to evaluate the collection of winter triticale (*Triticosecale*) by the polymorphism of alleles of *Wx* genes and the size of starch granules, as well as to establish the relationship between these traits. During research work were used PCR analysis, infrared spectrometry, light microscopy and different statistic methods. As result of study, it was found the presence of a wild-type allele of the *Wx*-B1 gene in all studied samples of winter triticale, eight of which had a null-allele of the *Wx*-A1 gene. The collection samples differed significantly in the size of the starch granules – the limits of variation ranged from 15.4 to 20.0 μm . It was determined that samples with low content of average sizes of starch granules were characterized by greater homogeneity of granulometric structure. However, no significant correlations were found between the size of the starch granules, the starch content and the allelic state of the *Wx*-A1 gene. After processing obtained data was selected collection specimens, that could be sources of valuable traits for new varieties suitable for processing into bioethanol.

Key words: bioethanol, amylopectin starch, sizes of starch granules, correlation analysis, sources of valuable traits.

INTRODUCTION

The **aim** of our study was to evaluate the collection of winter triticale (*Triticosecale*) by the polymorphism of *Wx* gene alleles and the size of starch granules of grain, to establish links between these traits. Main **significance** of this research is to identify collection specimens, that will be sources of valuable traits to create new varieties suitable for processing into bioethanol.

The main type of biofuel in the world is ethanol, the production of which is environmentally friendly and is relatively cheap (Chen-Guang *et al.*, 2019; Deepak end Vijay, 2019; Schneider *et al.*, 2018; Shreyas *et al.*, 2018; Sujit *et al.*,

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2019; Zabed *et al.*, 2017). In Ukraine, cereals are mainly used as raw material for bioethanol production (Brei end Shchutskyi, 2016). Researchers have proven the prospects for the use of triticale grain for biofuel production, the advantages of which are primarily due to high yields and unpretentiousness to growing conditions (Cristina *et al.*, 2016; Habtamu *et al.*, 2018; McGoverin *et al.*, 2011). In addition, triticale grain contains a lot of starch, which is characterized by increased enzymatic activity, which generally provides better cost-effectiveness of bioethanol production compared to other crops (Abdul *et al.*, 2020; Klikocka *et al.*, 2019; McKenzie *et al.*, 2014; Rybalka, 2012). To further increase the volume and efficiency of biofuel production from triticale grains, it is necessary to create and introduce new varieties that will meet the modern requirements for suitability for processing into bioethanol (Dumbravăa *et al.*, 2016; Grabovec *et al.*, 2015; Kour. *et al.*, 2019).

The yield of alcohol depends not only on the starch content in the grain, but also on the efficiency of its transformation into bioethanol, which is determined by the qualitative characteristics of starch. Amylopectin or waxy starch is characterized by high enzymeability, so cereals with starch of this type provide increased total yield of alcohol per unit mass and have advantages over use in the alcohol distillate industry (Yancheng *et al.*, 2013). There are currently no triticale wax varieties among those introduced into production. Also, for triticale varieties suitable for processing into bioethanol, the granulometric structure of starch is an important indicator, because grain with small and aligned starch granules due to the larger area of reaction with enzymes has an accelerated fermentation process (Rybalka *et al.*, 2013; Yaeel end Francisco, 2015; Yaeel, 2018). Thus, in the selection of triticale it is relevant and promising to create varieties with high content of amylopectin starch and with a fine and homogeneous composition of starch granules, which will increase the yield of bioethanol in grain processing (Rybalka *et al.*, 2018; Zhao *et al.*, 2009).

MATERIAL AND METHODS

The research was conducted during 2017–2019 at the National Scientific Center "Institute of Agriculture of NAAS", Ukraine. The soils of the fields belong to sod-medium-podzolic sandy loams. During the years of research, the temperature regime generally exceeded the value of the average long-term norm, and the amount of precipitation was characterized as arid.

The object of the study were 43 collection samples of winter triticale, which are represented by breeding numbers and varieties of selection of the Institute of Agriculture and varieties of foreign origin (Poland, Russia).

To determine the allelic state of the *Wx* gene by polymerase chain reaction (PCR), total DNA was isolated by CTAB + PVP. PCR was performed in an Applied Biosystems 2720 Thermal Cycler using a set of GenPak®PCRCore reagents in 20 µl of the reaction mixture containing 1 unit. Taq DNA polymerase (Somma *et al.*, 2006; Stewart *et al.*, 1993). When performing PCR analysis used the following primers:

gene Wx-A1 – Wx-A1F, 5`-ccccaagcaaagcaggaaac-3`.
Wx-A1R, 5`-cggcgtcgggtccatagatc-3`.
gene Wx-B1 – Wx-BDFL, 5`-ctggcctgtacctaagagcaact-3`.
Wx-BRC1, 5`-ggttgcggttggggtc gatgac-3`.
Wx-BFC, 5`-cgtagtaaggtgcaaaaaagtgccacg-3`.
Wx-BRC2, 5`-acagcctattgtaccaagaccatgtgtg-3`.

Detection of PCR results was performed by horizontal electrophoresis in 1.2% agarose gel in TBE buffer in the presence of ethidium bromide, followed by visualization of the results in an ultraviolet transilluminator (Brody end Kern, 2004). The size of DNA fragments was evaluated for motility compared to control DNA markers.

For analysis to determine the size of starch granules, several grains from each sample were ground in a laboratory grinder and 20–30 mg of obtained flour was taken, followed by staining in 2 ml of Lugol's iodine solution. The particle size distribution of the starch was examined by light microscopy in combination with digital analysis of the obtained images via a USB camera and *ImageJ* software (Wilson *et al.*, 2006; Starychenko end Levchenko, 2019).

Determination of starch content in the grain was performed by infrared spectrometry on the device Infracalc 1241. To confirm the statistical reliability of the obtained data were used various statistical methods using computer programs *Excel 2007* and *STATISTICA 8*.

RESULTS

The collection of winter triticale was analyzed by the allelic state of wax genes by PCR. The controls were soft winter wheat varieties: Sofiyka wax variety and Oksana variety with ordinary starch (wild type).

According to the results of molecular genetic analysis of 16 samples the amplicon 652 bp, which corresponds to the null-allele of the Wx-A1 gene, was found in breeding numbers triticale 229, 223 and 201 (tracks 7, 9 and 10) and in the control variety Sofiyka (15). Amplicons 495 and 176 bp were found in all other samples of winter triticale and wheat control variety Oksana, which confirms the presence of a wild-type allele of the Wx-A1 gene (Fig. 1). Among the following analyzed 29 collection samples, the presence of amplicon 652 bp (null-allele Wx-A1) was found only in varieties Lubomyr, Petrol, Poliskyi 7 and breeding numbers 141 and 153.

The results of the analysis of the winter triticale collection by the allelic state of the Wx-B1 gene showed that all triticale samples have an amplicon of 778 bp, which indicates the presence of a wild-type allele of the Wx-B1 gene.

To evaluate the collection of winter triticale by the granulometric size distribution of starch in the samples, the maximum, minimum and average sizes of starch granules were determined. The largest and smallest granules are usually present in small quantities, so an important characteristic of the particle size distribution is the average size of starch granules. It was found that the collection samples differed significantly in this indicator. Thus, in the variety Yasha the

average grain size of starch was 15.4 μm , and in the variety Almaz – 20.0 μm (Fig. 2).

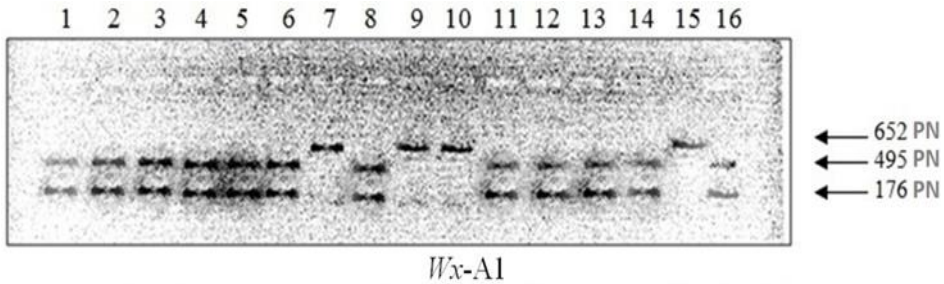


Figure 1. Electrophoregram of PCR of *Wx-A1* gene products in collection samples of winter triticale and control varieties of winter wheat

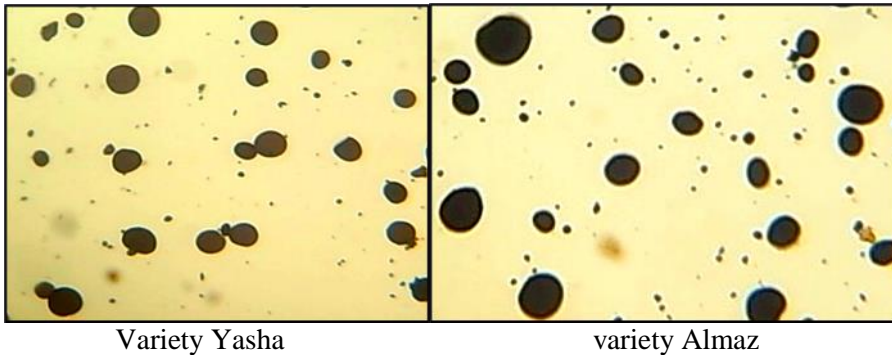


Figure 2. Granules of starch grain of winter triticale, microphoto at magnification x 45

The largest average sizes of starch granules were determined in such samples as Maetok Polissya, 215, Dokuchaevske, Almaz (18.1–20.0 μm), and the smallest – in samples Yasha, Mundo, 199, 141, 123 (15.4–15.9 μm). The number of samples with average sizes of starch granules up to 16.0 μm was 11.6% of the total collection, with a size from 16.1 to 17.0 μm – 41.8%, from 17.1 to 18.0 μm – 32, 6%. Groups of samples with average granule sizes of 18.1–19.0 μm and 19.1–20.0 μm accounted for 9.3 and 4.7% of the total collection.

According to the results of comparative evaluation of collection samples of triticale, no relationship was found between the size of starch granules and the content of starch in the grain. Such pairs of samples as Yasha and Mundo, 201 and 185, Petrol and Lubomyr, which were characterized by a close-sized particle size distribution, differed significantly in starch content (Fig. 3). The use of correlation analysis confirmed the lack of reliable relationships between these traits, which proves the possibility of selection work in the direction of simultaneously increasing the starch content and reducing the size of starch granules.

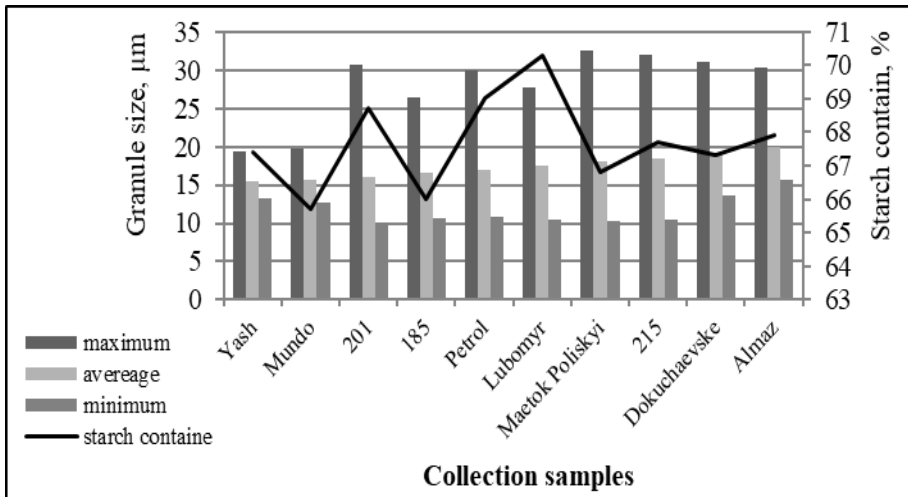


Figure 3. The size of starch granules and starch content in the grain of winter triticale collection samples, 2017–2019

Grain, the starch of which is homogeneous in granule size distribution, is more suitable for processing into bioethanol. Therefore, the analysis of starch samples is relevant not only for the size of its granules, but also for the alignment of the granule size distribution. It was found that in the varieties Yasha and Mundo starch consisted only of small granules up to 20.0 µm. Also, in the variety Poliskyi 7 and numbers 221, 201 and 199 revealed a relatively uniform particle size distribution of starch, where such granules ranged from 85.7 to 86.8%. In general, the collection shows a tendency to link the alignment of the granule size distribution and the average size of starch granules. Samples with large indicators of medium size differed in their greater heterogeneity compared to samples with small starch granules.

In the collection samples of winter triticale with null allele of the *Wx-A1* gene, a relatively small starch grain size was found, which averaged 16.5 µm in these samples. In sample 141 the average size of starch granules was 15.9 µm, in samples 201, Poliskyi 7, 229, 223 and 153 it varied from 16.1 to 16.6 µm and only in varieties Petrol and Lubomyr was 17.0 and 17.5 µm, respectively (Table 1). In varieties with the usual type of starch, such as Aristokrat, Solodyuk, Almaz and Dokuchaevske, the average granule size ranged from 18.0 to 20.0 µm, and the average for the collection of samples of triticale with wild-type *Wx-A1* – 17.3µm. Therefore, an assumption was made about the tendency to decrease the size of starch granules in partially amylopectin forms of triticale with a null allele of the *Wx-A1* gene.

To confirm the association of the allelic state of the *Wx-A1* gene with such quantitative traits as starch content and size of starch granules, a nonparametric method of calculating the Kendall correlation force was used (Khalafian, 2010).

Table 1. The average size of starch granules in collection samples of winter triticale with different allelic state of the *Wx* genes, 2017–2019

Collection sample	Country of origin	Allelic state of the <i>Wx</i> genes		Average size of starch granules, μm
		<i>Wx</i> -A1	<i>Wx</i> -B1	
Samples with partially amylopectin type of starch				
141	UKR	null-allele	wild type	15,9
201	UKR	null-allele	wild type	16,1
Poliskyi 7	UKR	null-allele	wild type	16,3
229	UKR	null-allele	wild type	16,4
223	UKR	null-allele	wild type	16,5
153	UKR	null-allele	wild type	16,6
Petrol	UKR	null-allele	wild type	17,0
Lubomyr	UKR	null-allele	wild type	17,5
Samples with the usual type of starch				
Arystokrat	UKR	null-allele	wild type	18,0
Soloduk	UKR	null-allele	wild type	18,0
Dokuchaevske	RUS	null-allele	wild type	19,1
Almaz	RUS	null-allele	wild type	20,0

Table 2. Correlation of amylopectin type of starch controlled by the *Wx*-A1 gene with starch content and size of starch granules in winter triticale

Quantitative signs		Year of the study	<i>Wx</i> -A1 alleles
The starch content in the grain		2017	-0,07
		2018	0,00
		2019	0,02
		average for 3 years	-0,01
The size of starch granules	maximum	2017	-0,15
		2018	-0,09
		2019	0,01
		average for 3 years	-0,08
	average	2017	-0,09
		2018	-0,03
		2019	-0,10
		average for 3 years	-0,06
	minimum	2017	-0,06
		2018	-0,20*
		2019	-0,21*
		average for 3 years	-0,19*

* Correlations are significant at $p < 0.1$

There are no significant correlations between amylopectin-type starch and starch content, which is confirmed by the results of studies separately for 2017–2019 and on average for three years (Table 2). There was also no significant

correlation between the allelic state of the *Wx*-A1 gene and the maximum and minimum sizes of starch granules. Weak negative significant correlation (at $p < 0.1$) was observed with the average size of starch granules of the samples in 2018, 2019 and on average over three years. The revealed regularities prove the possibility of creating amylopectin varieties of triticale with small starch granules.

The graphic image clearly showed a different range of variation of the average sizes of starch granules in the collection samples of winter triticale in groups with wild-type and null-allele gene *Wx*-A1 (Fig. 4).

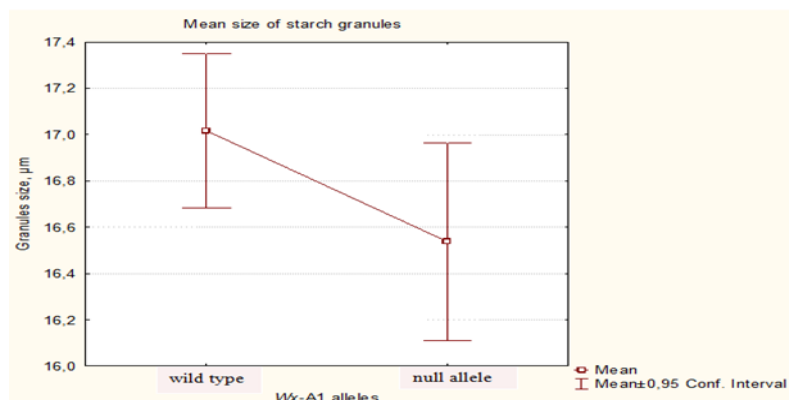


Figure 4. Ranges of distribution of average size of starch granules in winter triticale samples depending on the allelic state of the *Wx*-A1 gene.

Null-allele genotypes had a smaller average granule size than samples with the wild-type allele of the *Wx*-A1 gene, which was confirmed by a downward shift in the confidence interval in the group of samples with a partially amylopectival type of starch. The range of variation in the samples of the first group was 16.7–17.3 μm, and that of the second group was 16.1–16.9 μm. A larger range of variation was found in samples with a zero allele. Therefore, the dependence of the average size of starch granules on the allelic state of the *Wx*-A1 gene was confirmed.

DISCUSSION

The consumption of fuel and energy is increasing in the world. But fossil sources are being depleted and not renewed. Therefore, it is important to use renewable biological fuels, one of which is bioethanol. The source for the production of bioethanol can be triticale grain (Mupondwa *et al.*, 2018). The triticale varieties, that used for processing into bioethanol, should meet certain quality traits, including the content of starch and its quality.

Researchers have shown that the efficiency of processing grain into bioethanol also depends on the particle size distribution of starch (Cornejo-Ramirez *et al.*, 2015; Litvyak *et al.*, 2018). Thus, according to Rybalka (Rybalka

et al., 2013), Litvyak (Litvyak *et al.*, 2018) and others, it is established that the best efficiency of transformation of starch into alcohol have varieties of crops with the highest percentage of fine granules. It was found that the starch granules of different cultures differ in size, shape and surface morphology. Starch grains of regular and irregular oval, round and multifaceted shape, the sizes of which vary from 0.5 to 60.0 μm (Lindeboom *et al.*, 2004), have been identified. A number of scientists from the United States, China, Ukraine and other countries have shown that the size of starch granules differs not only within genera and species, but also different varieties of the same culture (Seok-Ho *et al.*, 2009; Zabolotets *et al.*, 2018). There is evidence that the size of the granules and starch content in different crops are interdependent: at high values of the average size of starch grains, the starch content decreases, and at lower values, the percentage of starch increases (Zabolotets *et al.*, 2018). According to the results of our research, a different genetically determined granulometric structure of starch in varieties and selection numbers of winter triticale was established. However, no relationship was found between the starch content in the grain and the size of the granules: collection samples with both high and low starch content were characterized by different particle size distribution.

Physico-chemical properties of amylopectin starch determine its advantages over conventional types of starch and the prospects for use in various industries, especially for the production of bioethanol (Chunyan *et al.*, 2020a; Chunyan *et al.*, 2020b; Fan, 2018; Yongfeng and Jay-lin, 2018). Varieties of wheat, corn, millet, sorghum and other crops with high amylopectin content have been obtained in the world, in which the synthesis of amylose is completely or partially blocked (Evžen and Dvořáček, 2017; Gago *et al.*, 2014). To identify new valuable sources of starch wax, scientists are widely conducting research to identify polymorphism of *Wx* genes in different cultures using polymerase chain reaction (Morhun *et al.*, 2015; Yuangen *et al.*, 2013; Zhirnova *et al.*, 2019). Three *Wx* genes have been identified in common wheat, each with several alleles (Juan and Carlos, 2016; Maningat *et al.*, 2009; Zhao *et al.*, 2009) – 77, 171, 185. According to Rybalka O. (Rybalka, 2015), in the triticale two recessive alleles of the genes *Wx-A1* and *Wx-B1* are localized in genome A, in genome B *Wx*-alleles are not detected. According to the results of the analysis of the winter triticale collection, we isolated 8 samples with the presence of the *Wx-A1* gene in the genome of null-alleles.

According to the results of research by a number of scientists in determining the granule size distribution of different types of starch revealed a tendency to reduce the size of the granules in amylopectin varieties of wheat. Amylose content has been shown to be negatively correlated with the percentage of fine granules in the starch and positively correlated with the percentage of large ones (Wenyang *et al.*, 2016; Xurun *et al.*, 2015). Our studies found a decrease in the size of starch granules in partially amylopectin samples of winter triticale and found a significant negative correlation between the presence of the *Wx-A1* allele in the samples and the average size of starch granules. In general,

we can say that the identification of patterns of variation in the size of starch granules and polymorphism of the gene pool of cultures by allelic state of Wx genes require further in-depth study to use the results in creating a new source material with a combination of genotypes biofuels.

CONCLUSIONS

Eight samples (varieties Lubomyr, Petrol, Poliskyi 7 and breeding numbers 141, 153, 201, 223, 229) with a null-allele by the Wx-A1 gene were isolated, which indicates a sufficient frequency of this allele in the gene pool of triticale common in Ukraine. It was found that all collection samples of winter triticale had a wild-type allele by the Wx-B1 gene.

It was proven the genetic diversity of the winter triticale collection by the granulometric structure of starch has been brought. Limits of variability for the average size of the granules were set from 15.4 μm in the variety Yasha to 20.0 μm in the variety Almaz. It has been established that from among the high indicators of the average size of starchy grains, a large heterogeneous stock of starch was grown.

A weak negative significant correlation (at $p < 0.1$, according to Kendall) was found between the presence of Wx-A1 allele samples and the average size of starch granules, which confirmed the observed tendency of decreasing starch grain size in partially amylopectin samples of triticale.

Selected varieties Yasha and Mundo, characterized by fine and homogeneous granulometric structure of starch (average granule size 15.4 and 15.6 μm) and samples 141, 201, 229, 223, 153, Poliskyi 7 with partially amylopectin starch and medium granule size from 15.9 to 16.6 μm are valuable sources for creating varieties for alcohol-distillate use.

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Nikitovic, J., Andrijasevic, D., Krajisnik, T., Antic, M., Kajkut Zeljkovic, M., Samardzic, S., Stojanovic, M. (2021): Morphometric measures of the Gatacko cattle on the territory of Gacko municipality. *Agriculture and Forestry*, 67 (3): 159-166

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MORPHOMETRIC MEASURES OF THE GATACKO CATTLE ON THE TERRITORY OF GACKO MUNICIPALITY

SUMMARY

Gatacko cattle, as one of the indigenous breeds of the Bosnia and Herzegovina, namely its entity of Republic of Srpska, deserve special attention when it comes to detailed description of the breed, inventory and genotyping. This kind of research is absolutely necessary in order to establish programs for *in situ* preservation of genetic resources. As the name of the breed suggests, Gacko municipality is the most relevant for this breed in terms of breeding and the total number of cattle, and was therefore selected for our research.

The aim of this paper was to determine morphometric parameters of the Gatacko cattle by conducting appropriate measurements. The study involved 288 cows, and the total of 15 parameters were measured on each cow. The analysis of obtained data was performed using methods of descriptive statistical analysis. Also, the phenotypic correlation between the morphometric parameters was calculated.

The results indicates higher values of all morphometric parameters, except for the shin girth. The contribution of this research is reflected in a large number of samples on which morphometric characterization were performed and correlations determined, which provide material for further research towards genotyping of this indigenous breed and establishing the breeding goals.

Keywords: Gatacko cattle, morphometric parameters, phenotypic correlation

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INTRODUCTION

The homeland of Gatacko cattle is a mountainous region of Gacko municipality in East Herzegovina, with average altitude of more than 1000 m, with continental climate and average temperature of just 8°C. At the time when Bosnia and Herzegovina were annexed by Austro-Hungarian monarchy (1878), the cattle breeding in Herzegovina was based on Busha cattle, a small cow with a mass of just 200 kg, but resilient and well adapted to climate. As the new government wanted a larger cattle, with better production of milk, but still able to withstand harsh condition, it was decided to cross-breed the Busha cattle with Viptal and Oberintal cattle, to create a new breed, the Gatacko cattle.

The Gatacko cattle are short-legged cattle, short-headed, with wide and uneven forehead. The muzzle is darkly pigmented with light edge. The horns are thin and pointed forward. Basic color is gray, but it can also be brown with dark shading on certain parts of the body. Cows have body mass around 400 kg, while bull's weight is around 750 kg. The Gatacko cattle are most valued for its milk production, which can go up to 2,500 l per year (Katica *et al.*, 2004).

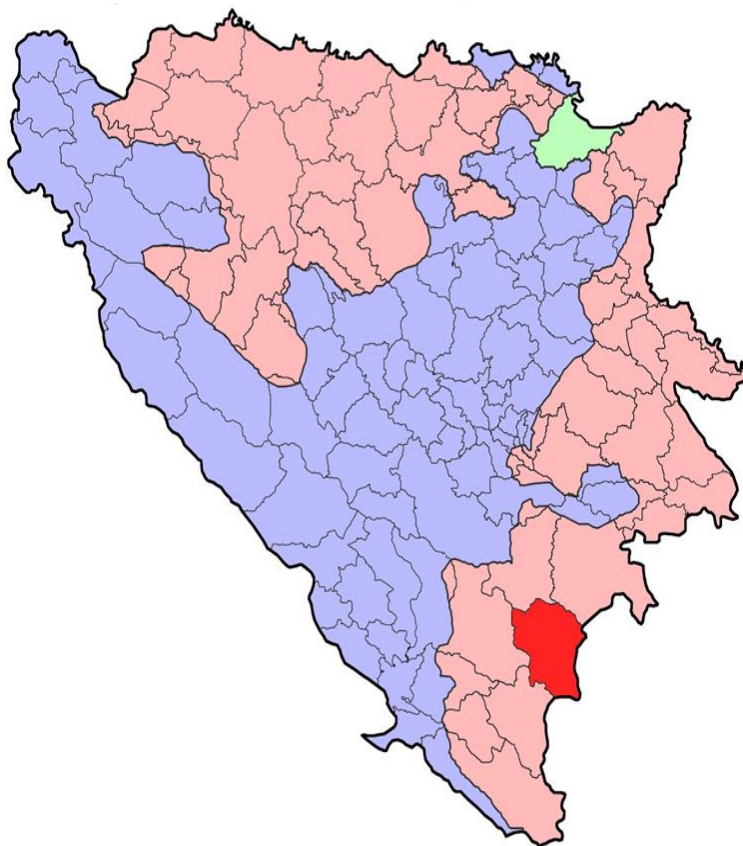
Considering that the overall decrease of cattle population has been recorded in the Republic of Srpska and Bosnia and Herzegovina, it is quite understandable that the same has happened regarding indigenous breed of Gatacko cattle (The Law on Cattle Breeding, Official Gazette of the Republic of Srpska, No 44/15, article 40, and paragraph 2). The importance of indigenous breeds of domestic animal is indisputable. These breed, and thus the Gatacko cattle, influence the revitalization of rural areas by generating additional income for the local population. As the Gatacko cattle are well adapted to the local climate and terrain, they help maintain pasture areas, preventing the devastation and succession of habitats (biotopes).

The Gatacko cattle is recognized as endangered breed, therefore it is absolutely necessary to conduct inventory and morphometric measurements of potentially autochthonous individuals, followed by genotyping. All individual cows and bulls, confirmed as indigenous, will be involved in project of conservation of Gatacko cattle in order to preserve its gene pool. Morphometric measurements presented in this paper are part of broader activities aimed on revitalization of Gatacko cattle.

MATERIAL AND METHODS

The aim of this paper was to determine morphometric parameters (15 of them) of indigenous breed Gatacko cattle, on the area of municipality Gacko, Republic of Srpska – Bosnia and Herzegovina. Additional aim was to determine the variability of the mean value of examined parameters, indices of body measures in relation to the height at withers, correlation between the examined parameters and the significance of the calculated correlation coefficients. The morphometric measurements, for purpose of the morphometric characterization of the Gatacko cattle, was performed on 288 cows, all females, as there were only

few bulls found in the field. Moreover, the morphometric measurements of the bulls are quite different to cows, so they were omitted in this research. Based on the registry (ear tag), and the statement of the owners, the age of each cow was defined, and no cow was younger than 2 years at the time of this research. The measurements were conducted at the beginning of the year 2021 on the following localities: Miholjace, Vratkovici, Stari Dulici, Novi Dulici, Dobrelja, Gareva, Samobor, Mala Gareva, Domrke, Gradina, Mume, Lukovice, Medanici, Nadanici, Stepen, Przine, Cernica, Danici, Krvarevo and Fojnica, all belonging to municipality Gacko.



Picture 1. The territory of municipality Gacko (marked red) on the map of Bosnia and Herzegovina and Republic of Srpska (marked pink)

The following morphometric parameters were measured on each cow: Height at withers (HW), back height (BH), loin height (LH), body length (BL), chest width (CW), chest depth (CD), chest girth (CG), hip width (HW), pin bones

width (PBW), front shin girth (FSG), back shin girth (BSG), head length (HL), forehead width (FW), horn length (HNL), horn girth (HNG). Morphometric measurements were performed using ribbon and Lydtin's rod (Lalovic and Zdralic, 2018).

In addition to absolute body measures, the relative indicators of physical development, ie indices, were calculated. The body indices represent the ratio of one measured body parameter to another, in this case to the height at withers. The indices were calculated according to Alderson (1999). The obtained results were processed using the software Microsoft Office Excel 2010, and the phenotypic correlation between the morphometric parameters was determined (R Core Team, 2015). Descriptive statistic values were calculated: coefficient of variation (CV), standard error (SE), standard deviation (SD), minimum value (MIN), maximum value (MAX), mean value (\bar{x}) and indices in relation to height at withers (I). As statistically significant were taken differences $P < 0.05$.

RESULTS AND DISCUSSION

A phenotypic characterization of any breed can not be done without morphometric characterization. Performing this characterization is necessary in order to bring decisions about development of the breed and its breeding programs (FAO, 2012). Morphometric characterization of the Gatacko cattle is conducted on 15 body parameters.

Values of morphometric parameters in examined Gatacko cattle are shown in Table 1.

Table 1. Average variability of body parameters in Gatacko cattle

Parameter (in cm)	$\bar{x} \pm SD$	SE	CV	I*	Min	Max
Height at withers (HW)	133.00±2.99	0.18	2.25	1.00	120.00	156.00
Back height (BH)	131.80±3.05	0.18	2.31	0.99	119.00	155.00
Loin height (LH)	132.60±2.89	0.17	2.18	0.10	120.00	155.00
Body length (BL)	156.40±5.25	0.31	3.36	1.18	130.00	173.00
Chest width (CW)	64.30±5.65	0.33	8.79	0.48	42.00	72.00
Chest depth (CD)	78.40±5.70	0.34	7.27	0.60	45.00	88.00
Chest girth (CG)	183.60±6.66	0.40	3.63	1.38	165.00	233.00
Hip width (HW)	61.40±2.20	0.13	2.80	0.46	47.00	73.00
Pin bones width (PBW)	42.90±2.77	0.16	6.46	0.32	23.00	51.00
Front shin girth (FSG)	13.70±0.99	0.06	7.23	0.10	12.00	20.00
Back shin girth (BSG)	13.10±1.00	0.06	7.63	0.09	10.00	18.00
Head length (HL)	47.50±1.78	0.10	3.75	0.36	40.00	52.00
Forehead width (FW)	24.20±1.66	0.09	6.86	0.18	14.00	29.00
Horn length (HNL)	13.80±2.47	0.15	17.40	0.10	7.00	25.00
Horn girth (HNG)	11.80±1.48	0.09	12.54	0.09	9.00	16.00

*Indices in relation to the height at withers

The average height at withers was 133.00 ± 2.99 , and interval of variation between 120 cm and 156 cm. Those values are similar to research by Varatanovic (2018) who examined three different groups of cow and found the mean value of this parameter 5.59 cm, 5.92 and 2.88 cm different to our research. Gutic *et al.* (2003) in their research on 125 cows found this parameter only 122.71 cm on average. The mean value for back height on our examined Gatacko cattle was 131.80 cm with standard deviation (SD) of 3.05 and variation interval from 119 cm to 155 cm, while the coefficient of variation (CV) was 2.31%. When it comes to loin height, mean value was 132.60 cm, with interval of variation from 120 cm to 155 cm, and coefficient of variation 2.18%. The loin height in the research of Varatanovic (2018) on three groups was similar to our results (128.83 cm, 130.88 cm and 134.00 cm). Comparing to the work of Ilancic (1952) and Gutic *et al.* (2003), we can conclude that our results for this parameter are around 10 cm higher. The average body length in examined cows was 156.40 cm, with variation interval from 130 cm to 173 cm, and coefficient of variation 3.36%. Gutic *et al.* (2003) found this parameter slightly higher with average value of 159.30 cm and interval of variation from 144 cm to 171 cm, while Varatanovic (2018) found body length at 142.56 cm on average. The mean value for the chest depth in our examined cows was 78.40 cm with interval of variation from 45 cm to 88 cm. Gutic *et al.* (2003) found the mean value of this parameter at 67.45 cm, which is significantly lower than our results. The chest girth in our research was 183.60 cm on average, which is almost identical to Varatanovic (2018), and 14.71 cm higher than Gutic *et al.* (2003). Other authors also found lower results for this parameter. Pajanovic (1961) compared this parameter for three different locations, and found that cows from Gacko have chest girth 151.41 cm on average, from Nevesinje 147.25 cm, and from Kalinovik 148.07 cm. Sucic *et al.* (1978) found the mean value of this parameter at 160.70 cm, while Popovic *et al.* (1979) found the mean value of 166.20 cm. The hip width in our examined cows was 61.40 cm on average, which is 9.36 cm higher than value found by Gutic *et al.* (2003). When it comes to the front shin girth we have found mean value at 13.70 cm, while the mean value of the back shin girth was 13.10 cm. Morphometric parameters of the head was on average 47.50 cm for the head length, 24.20 cm for the forehead width, 13.80 cm for the horn length and 11.80 cm for the horn girth. Coefficients of variation (CV) for 13 out of 15 measured paremeters ranged from 2.18% (loin height) to 8.79% (chest width). Only the horn length (17.40%) and horn girth (12.54%) have higher coefficient of variation, which can be explained by cow`s minor moving during measurements, and should be taken with reserve when making conclusions about this parameters.

The correlation of average values of examined body parameters, as well as statistical significance of correlations is shown in table 2.

Height at withers (HW) is in very highly positive correlation ($P < 0.001$) with all examined parameters, except for the back shin girth ($P < 0.05$), and horn length and girth, where the correlation is highly significant ($P < 0.01$).

Table 2. Phenotypic correlations of morphological characteristics of the Gatacko cattle

	BH	LH	BL	CW	CD	CG	HW	PBW	FSG	BSG	HL	FW	HNL	HNG
HW	0.960***	0.940***	0.560***	0.250***	0.260***	0.610***	0.480***	0.310***	0.260***	0.150*	0.200***	0.240***	0.170**	0.160**
BH		0.914***	0.564***	0.322***	0.315***	0.595***	0.465***	0.302***	0.210***	0.083 ^{ns}	0.182**	0.250***	0.151*	0.114 ^{ns}
LH			0.526***	0.153**	0.174**	0.568***	0.466***	0.285***	0.308***	0.212***	0.259***	0.149*	0.183**	0.157**
BL				0.387***	0.430***	0.551***	0.511***	0.255***	0.155*	0.028 ^{ns}	0.361***	0.229***	0.131*	0.027 ^{ns}
CW					0.576***	0.310***	0.310***	0.274***	-0.122*	-0.269***	0.248***	0.210***	0.079 ^{ns}	-0.008 ^{ns}
CD						0.274***	0.361***	0.298***	-0.055 ^{ns}	-0.144*	0.225***	0.324***	0.021 ^{ns}	0.016 ^{ns}
CG							0.481***	0.321***	0.352***	0.195**	0.180**	0.282***	0.106 ^{ns}	0.140*
HW								0.404***	0.328***	0.202***	0.408***	0.374***	0.160**	0.203***
PBW									0.133*	0.040 ^{ns}	0.111 ^{ns}	0.351***	0.110 ^{ns}	0.279***
FSG										0.784***	0.043 ^{ns}	0.091 ^{ns}	0.103 ^{ns}	0.202***
BSG											-0.057 ^{ns}	-0.003 ^{ns}	-0.040 ^{ns}	0.113 ^{ns}
HL												0.196**	0.175**	0.036 ^{ns}
FW													0.116 ^{ns}	0.090 ^{ns}
HNL														0.235***

^{ns} P > 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001

Back height (BH) is in very highly significant correlation ($P < 0.001$), highly significant ($P < 0.01$) or significant ($P < 0.05$) with most body parameters, while the significance was not confirmed ($P > 0.05$) in correlation with back shin girth and horn girth. We can conclude the similar correlation of body length (BL), hip width (HW) and horn length (HNL) with other examined parameters in this paper. Loin height (LH) has statistically significant correlation with all examined parameters, at every level of significance. Chest width (CW) has correlation significance at level $P < 0.001$ with most parameters, while the correlation to front shin girth (FSG) and back shin girth (BSG) is negative at levels $P < 0.05$ and $P < 0.001$, respectively. Similar results were found at correlations between chest depth (CD) and examined parameters, except for front shin girth (FSG) where no significance was found ($P > 0.05$). Also, for both of these parameters (chest depth and width) the statistical significance to horn parameters (length and girth) was not confirmed ($P > 0.05$). Chest girth (CG) has statistically positive significant correlation with all examined parameters, at every level of significance, except to horn length (HNL), where significance was not confirmed ($P > 0.05$). Pin bones width is in statistically significant correlation front shin girth, forehead width and horn girth at levels $P < 0.05$ and $P < 0.001$, while statistical significance was not confirmed in correlation to back shin girth, head length and horn length ($P > 0.05$). Front shin girth is in very highly significant correlation ($P < 0.001$) with back shin girth and horn length, while the correlation of back shin girth to head length, forehead width and horn length are all negative and statistically not confirmed ($P > 0.05$). Head length is in highly significant correlation ($P < 0.01$) to forehead width and horn length, while the correlation of forehead width to horn parameters is not statistically confirmed ($P > 0.05$).

CONCLUSIONS

A detailed morphometric description of Gatacko cattle is very important as a precondition to further analysis of the breed, genotyping, establishing the breeding program and setting the breeding goals. We have measured 15 body parameters on 288 cows from the territory of Gacko municipality in the Republic of Srpska - Bosnia and Herzegovina. We have also determined the variability of examined parameters, and the significance of correlation between the measured parameters.

Based on the obtained results from our research on Gatacko cattle, we can conclude that all body parameters have higher values (except for shin girth) than previous examinations of other authors. The reason for this may be found in mating selection, which is commonly based on phenotype characteristics of the cattle, and under the man`s influence. Other reasons could be found in total number of examined cows, different breeding conditions and climate change in the last 50 years, when the previous research were performed.

The results from our research can be used for further detailed description of this breed.

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THE MOST IMPORTANT METHODS OF DISINFECTION IN BEEKEEPING

SUMMARY

The hygiene of beehives is of the utmost importance for the protection of the health of bees and bee products. Disinfection is a very important hygienic measure that is carried out in order to prevent and suppress infectious diseases of bees and contamination of honey and other bee products with harmful microorganisms. The disinfection procedure is very complex, and its efficiency depends on a large number of factors. In beekeeping, as well as in other branches of animal husbandry, preventive, liquid and final disinfection is carried out. During the disinfection, the following factors must be taken into account: the spectrum of action of the disinfectant, the method of application, the concentration of the disinfectant, the exposure time, the properties of the environment to be disinfected. The active substances of disinfectants usually affect the metabolism of microorganisms. For disinfection in beekeeping can be used: chlorine preparations, iodine preparations, bases, acids, quaternary ammonium compounds, aldehydes (glutaraldehyde, glyoxal), oxidizing agents (hydrogen peroxide, acetic acid). There is no ideal disinfectant. Due to the toxicity and other harmful effects of disinfectants, and often their insufficient efficiency, preference should be given to physical methods of disinfection, whenever possible. Chemical disinfection is often not successful, especially when it comes to the causative agents of American bee brood plague, which form very resistant spores. In that case, the only way is to burn the hives, so that they are not a source of infection. Against the spores of *Paenibacillus larvae*, the most effective chemical disinfectants are chlorine preparations, warm solution of sodium hydroxide, oxidizing agents, therefore, these disinfectants are most often used in beekeeping. However, the negative properties of these disinfectants should also be taken into account.

Key words: beekeeping, hygiene, disinfection, spores, *Paenibacillus larvae*

INTRODUCTION

Hygiene is of the greatest importance for the protection of the health of bees and bee products. That is why disinfection in beekeeping is being paid more

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and more attention. The process of disinfection destroys microorganisms by physical, chemical or combined procedures in order to stop the spread of infection from its source to the susceptible organism. Disinfection can be defined as the destruction or killing of microorganisms on objects, in water, in the air and on undamaged skin up to the number that will not be able to cause disease or spoilage of food. Sterilization, unlike disinfection, involves killing all microorganisms, both their vegetative and sporogenic forms. The term disinfectants mean chemical agents that are used to destroy microorganisms on surfaces, objects, utensils and other inanimate matter (Bojanić Rašović *et al.* 2016; Bojanić Rašović 2018a; 2019a; 2019b; 2019c; 2021a, 2021b, 2021c, 2021d).

MATERIAL AND METHODS

Disinfection in beekeeping

Types of disinfection in beekeeping

When considering a particular epidemiological situation, the following types of disinfection are distinguished: preventive, focal and final. Preventive disinfection is performed before an infectious disease appears in the apiary. This disinfection aims to prevent the occurrence of infectious diseases of bees. Preventive disinfection is carried out with disinfectants with a wide spectrum of action - agents that act on the largest number of microorganisms. It aims to reduce the number of all microorganisms that can be found in the apiary and that can lead to infectious diseases. Focal disinfection aims to kill the pathogen at the site of the outbreak and stop its further spread. It is carried out for the duration of the disease and is directed at the microorganism that led to the infectious disease. Final disinfection is carried out after the cessation of the disease, i.e. when two incubation periods have passed since the appearance of the last symptoms of the disease. The goal of final disinfection is to destroy all remaining infectious agents that have not been destroyed by focal disinfection. It is carried out with the same means and the same concentration as the focal disinfection (Radenković Damjanović, 2010, Bojanić Rašović, 2021b). Only approved preparations and procedures may be used for disinfection. During disinfection, the following must be taken into account: source, route, mode of spread of infectious disease, properties of microorganisms against which disinfection is performed, spectrum of action and other properties of disinfectant, method of application and required disinfectant concentration, exposure time, disinfecting environment, etc. It should also be borne in mind that the two disinfectants may have an inhibitory or potentiating effect (synergy) (Asaj, 2000, Vučemilo, 2008, Titera 2009).

Physical methods of disinfection

Physical methods of disinfection are mainly based on the use of dry or wet heat and the application of radiation. Incineration is the oldest and very good method of physical disinfection. This kills very resistant bacteria (such as the causative agent of the American bee brood plague). *Burning* is a procedure that involves exposing contaminated objects to flames, in order to destroy the

pathogens that are on them. In this way, metal objects as well as some wooden objects can be disinfected. The effect of disinfecting *hot water* under normal atmospheric pressure for 30 minutes is increased by adding 1-2% crystalline soda (Na_2CO_3 , sodium carbonate). Disinfection with hot water steam created by fixed or mobile steam generators is also a successful method of disinfection. Unlike water, water vapor can have temperatures higher than 100°C . In order for disinfection to be effective, water vapor must be under a certain pressure and at a minimum temperature of 110°C for 40-45 minutes. *Dry hot air flowing*, temperatures from 110°C to 150°C in ovens and dryers for 30 minutes are also used for disinfection (Bojanić Rašović, 2021a). *Ultraviolet radiation* with a wavelength of 253 - 280 nm has a bactericidal effect. Maximum efficiency is achieved with rays of wavelength 265 nm. Sources of UV radiation, in addition to the sun, are various fluorescent lamps. Ultraviolet radiation cannot penetrate inside the objects being treated - the radiation only acts on the surface being irradiated. Sporulating bacteria are resistant to ultraviolet radiation (Belojević, 2013, Titera 2009).

Chemical methods of disinfection

Disinfectants represent a diverse group of chemical substances that create unfavorable conditions for the survival of microorganisms. Microbicides (bactericides, fungicides, virucides, sporocides) kill microorganisms or their spores, while microbistatics (bacteriostatics, fungistatics) only stop the growth of microorganisms. The active substances of disinfectants usually affect the metabolism of microorganisms (Buchrieser and Miorini, 2009). Chemical reactions on which the action of disinfectants is most often based are: oxidation (chlorine and chlorine preparations - hypochlorites, chloramines, isocyanurates, iodine and iodine preparations - iodophores, hydrogen peroxide, other peroxide compounds, ozone, ethylene oxide), hydrolysis (acids, caustic soda, hot water), formation of protein salts (alkali metal and heavy metal salts), coagulation of proteins in microorganism cells (quaternary ammonium salts, metals, phenols, alcohols), changes in cell membrane permeability, mechanical damage to microorganism cells (quaternary ammonium salts), penetration into the enzyme system of microorganisms (metals, formaldehyde, phenols). Chemical methods of disinfection dominate over the physical methods in many ways. During the disinfection, it is necessary to carry out preliminary disinfection, which are mechanical cleaning and sanitary washing, and then the disinfection itself. Sometimes both phases can be performed using detergents and disinfectants. The effects of disinfection also depend on the way in which the disinfectant is applied to the places where microorganisms need to be destroyed. Disinfection of surfaces and objects can be done by immersion in disinfectant, wiping with a cloth soaked in disinfectant, spraying, releasing gas, evaporation of disinfectant. When performing disinfection, the contractor must have a protective suit, hat, gloves, boots, goggles and respirator, a handy pharmacy with appropriate antidotes (Vukićević and Hrgović, 1988, Asaj, 2000). The disinfectant solution should be prepared immediately before use, as its effectiveness declines over

time. Increased efficiency of some preparations can be achieved by higher temperature of the solution. For chlorine compounds, phenols and quaternary ammonium salts, the solution should be heated to 50 to 60 ° C, and for hydroxides to 80 ° C. It is very good to change disinfectants periodically, to prevent the formation of resistance of microorganisms to any of the active disinfectant substances.

RESULTS AND DISCUSSION

Types of chemical substances with disinfectant properties

The mechanism of action of *halogen* is based on a combination of the action of highly reactive halogen compounds and oxygen released in an alkaline environment. Sodium hypochlorite (NaClO) has a very good bactericidal and virucidal effect. Its use is wide. It is an effective ingredient in many commercial preparations. Commercially available preparations generally contain about 5% sodium hypochlorite (bleach), which are diluted according to the instructions. It needs to act for approximately 30 minutes to achieve a disinfectant effect (Titera, 2009). The hives are disinfected with a warm 0.5% hypochlorite solution for 20 minutes (Anon, 2014). Caution should be exercised when mixing sodium hypochlorite with acids and acidic disinfectants, as it releases hydrogen chloride gas which, when inhaled, has an irritating and toxic effect on humans. Chlorinated lime - calcium hypochlorite should contain at least 25% active chlorine. Solutions of *bases* and alkaline salts act on the basis of a high concentration of hydroxyl anions (OH⁻). Solutions with a pH greater than 12 are very effective. They can be used while they are hot, but then needs to be more careful. Caustic soda (NaOH) burns are very deep. Caustic soda solutions dissolve beeswax and fat, so they are widely used in beekeeping. They are easily available and relatively cheap. This group includes potassium hydroxide (KOH), sodium hydroxide (caustic soda, NaOH), calcium hydroxide (slaked lime, Ca(OH)₂ - used in a concentration of 10 to 20% for disinfection of soil, waste storage, etc.), sodium carbonate. *Sodium carbonate* (soda, Na₂CO₃) is used in a concentration of 2 to 6%, preferably as hot solutions (they destroy the spores of the causative agent of American bee brood at 80 ° C). Caustic soda is used to disinfect hives in a concentration of 2 - 4% (Periškić *et al.* 2013). *Inorganic acids* are relatively effective, but they are also caustic - they corrode materials. They act as aqueous solutions based on a high concentration of reactive hydrogen ions. They are not often used in beekeeping. Hydrochloric acid at a concentration of 10% (HCl) is used for general cleaning. Phosphoric (H₃PO₄) and sulfuric acid (H₂SO₄) in concentrations of 0.5 to 5% are used in beeswax processing. *Organic acids*, formic and oxalic are mostly used in beekeeping against the parasitic mite *Varroa destructor*. They also have a disinfecting effect against some bacteria and fungi (the cause of stone bee litter, lime bee litter and bee nosemosis). They are most often used in the form of steam (Anon, 2014). *Peracetic acid* (also known as peroxyacetic acid is a good agent for cleaning and disinfecting glassware. It is used in concentrations of 0.2 - 0.5%, where the treatment should last at least 10 minutes For disinfection in beekeeping practices can be used oxidizing substances. *Hydrogen peroxide* (H₂O₂) is stored as an aqueous solution. It is sold

as a 3%, 10% as well as a 30% solution. Solutions of 0.5 to 3% concentration are most often used for disinfection. *Organic peroxides* are modern, but expensive disinfectants. They work against the spores of the bacterium that causes the American bee brood plague (*Paenibacillus larvae*). Their advantage is that they are environmentally friendly because they decompose into harmless substances (Asaj, 2000, Titera, 2009). Some *metals* have a very toxic effect on living cells of microorganisms. Silver compounds are used for disinfection of drinking water. Bees tolerate colloidal silver, nanosilver and copper relatively well. Many commercial disinfectants are based on *alcohols* (ethanol, isopropyl alcohol and others). They have the best effect in aqueous solutions in a concentration of about 70% and in combination with other active substances. Concentrated alcohol (pure alcohol, 99%), as well as too dilute alcohol (eg concentrations of alcohol in rum, whiskey, plum brandy) do not have a good disinfectant effect. Alcoholic preparations are not enough to destroy the spores of microorganisms, nor the spores of the causative agent of the American bee brood plague. They are effective against some viruses that have a lipid envelope. The disinfecting effect of *aldehydes* is based on reduction reactions, as well as on their reaction with cell proteins of microorganisms, whereby protein denaturation occurs. Formaldehyde (HCHO) is a gas. An aqueous solution with a concentration of 35 to 40% is known as formalin. Very strict rules regulate its use, because it has a detrimental effect on human health (potential carcinogen). A 1-5% formalin solution is used to disinfect the surface (Anon, 2019). Porous objects such as wood and wax frames must not be disinfected with formaldehyde, as its residues cannot be removed after treatment. This dangerous substance must not come into contact with food and bee products (Žarković, 1971). Glutaraldehyde is the basis of very effective disinfectants. After prolonged exposure, it safely destroys bacteria, viruses and spores of microorganisms. It is used as part of mixed disinfectants. Before use, it is diluted in a concentration of 2% and alkalized by the addition of 0.3% NaHCO₃ (sodium bicarbonate). *Cyclic compounds* are group of substances that damage the enzymes and cell protoplasm of microorganisms. They irritate the skin and have a characteristic odor. Therefore, despite their effectiveness, they are not used in beekeeping. Some, like phenol, are carcinogenic. *Detergents* are complex in composition and can contain more than 25 different ingredients. Surfactants are the most important ingredients in detergents and are found in all types of detergents. Surfactants are substances that lower the surface tension of a liquid (water). Soaps are anionic surfactants, which are used for washing and cleaning, giving a surface-active anion in aqueous solution. The primary function of soap is to regulate the foam in laundry detergents, as it gives the greatest volume. Anionic surfactants make up 70 - 75% of the total surfactant consumption in the world. Quaternary ammonium compounds (benzalkonium chloride, cetrimonium chloride, etc. - commercial preparation Asepsol, 5% benzalkonium chloride solution) are cationic surfactants that act on gram positive bacteria - do not act on gram negative bacteria. Amphoteric surfactants (amphotensides) (lauryl diethylene diamino glycine - commercial preparation DeSu) - anionic groups are carriers of detergent and cationic carriers of bactericidal properties. The mechanism of action is based on damage to the cell wall of microorganisms, entry into the cell and disruption of its metabolism. They

have bactericidal, virucidal and fungicidal action. Due to its positive properties, biodegradability, anticorrosiveness, cleaning and deodorizing ability, microbicidal action, DeSu preparations are successfully used in disinfection (Anon (1), Papić, 2015). In beekeeping, a combination of *physical and chemical methods* is successfully used for disinfection. Cooking tools and utensils in hot water with the addition of 1-2% crystal soda (sodium carbonate, Na_2CO_3) or the addition of 3% NaOH is an effective method of disinfection.

The effects of chemical disinfectants on different groups of microorganisms are shown in Table 1.

Table 1. Effects of chemical disinfectants on different groups of microorganisms (Titera, 2009, Žarković, 1971).

Disinfectant	Effect on microorganisms				
	Bacteria	Spore	Fungi	Viruses	Application
Oxidizing agents (hydrogen peroxide)	Bactericid	Sporocid	Fungicide	Virucid	Skin, mucous membranes, surfaces, tools, instruments
Halogens (Chlorine, iodine and their compounds)	Bactericid	Sporocid	Fungicide	Virucid	Chlorine: surfaces, water Iodine: skin, mucous membranes
Alcohols (ethyl alcohol, isopropyl alcohol)	Bactericid	No effect	Fungicide	Virucid	Skin, mucous membranes, surfaces, instruments
Aldehydes (glutaraldehyde, formaldehyde)	Bactericid	Slightly sporocidal	Fungicide	Virucid	Surfaces, instruments
Phenols	Bactericid Bacteriostatic	No effect	Fungicid	Virucid	Skin, mucous membranes, surfaces, tools, instruments
Ethylene oxide	Bactericid	Sporocidal	Fungicid	Virucid	Surfaces, instruments, tools, thermo-stable drugs, food
Detergents	Bactericid	No effect	Fungistatic	No effect	Cleaning of metal and wooden surfaces, washing and cleaning of packaging, washing dishes Skin, mucous membranes
Biguanide (chlorhexidine)	Bacteriostatic	Biguanide (chlorhexidine)	Fungistatic	Virostatic (act on enveloped viruses)	Skin, mucous membranes
Inorganic acids	Bactericid	Sporocid	Fungicid	Virucid	Disinfection of work surfaces, corrosion resistant floors
Organic acids	Bactericid to some bacteria	No effect	On some fungi	On some viruses	Disinfection of work surfaces, objects
Bases	Bactericid, especially on gram negative bacteria	Sporocid in hot solutions heated to 80-90°C	Fungicid	Virucid	Disinfection of work surfaces, objects

Table 2. shows the advantages and disadvantages of the most commonly used disinfectants

Table 2. Advantages and disadvantages of the most commonly used disinfectants (Anon. 2003).

Disinfectant	Advantages	Disadvantages
Alcohols	They act quickly, leave no residue, no paint	Volatile, can harden rubber objects, dissolves glue, toxic
Chlorine and chlorine compounds	Cheap, fast acting, easily available	Corrosive to metals, inactivated by organic matter, irritating to skin and mucous membranes, should be used in well-ventilated rooms, their shelf life is shortened after dilution
Formaldehyde	Active in the presence of organic matter	Very limited use, Carcinogenic, poisonous, very irritating, strong odor
Glutaraldehyde	Good disinfectant for equipment and accessories that cannot be sterilized by heat (2% solution), non-corrosive, active in the presence of organic matter	Extremely irritating and toxic to skin and mucous membranes, expensive, its shelf life is shortened after dilution
Hydrogen peroxide	Strong oxidant, acts quickly, decomposes quickly to water and oxygen	Can be corrosive to aluminum, copper, brass or zinc, surface active - with limited penetration
Iodophors	They act quickly, relatively little toxic	They are corrosive to metals, can damage tissues, dyed fabrics, etc.
Peracetic acid	Suitable for items and utensils that cannot be disinfected by heat. Decomposes into water, oxygen, acetic acid, hydrogen peroxide). It acts quickly at low temperatures, it is active in the presence of organic matter	It can be corrosive, unstable when diluted
Phenols	For disinfection of walls, floors, objects, it is added to detergents and thus washing and disinfection are performed at the same time.	It leaves residues on surfaces, it is not used for surfaces that come in contact with food, it can be absorbed by leather and rubber, synthetic floors can become sticky if the use of phenol is repeated.
Quaternary ammonium compounds	For cleaning floors, walls, objects, usually do not irritate hands, usually have detergent properties, are not corrosive	It is not used for disinfection of instruments and tools, because it has a limited antimicrobial effect
Bases	Easily soluble in water, easily reaches microorganisms, decomposes organic matter, efficiency increases to 80-90°C	Corrosive, hard water reduces their efficiency, in contact with air NaOH is inactivated, causes burns, eye damage
Inorganic acids	In a very short time, they break down proteins and carbohydrates in the cell of microorganisms.	They irritate the skin and mucous membranes, cause burns, are corrosive, so they are rarely used
Organic acids	They are used as preservatives in the food industry	They do not have a strong bactericidal effect, they are corrosive

Most of the bee viruses known so far belong to small, single-stranded RNA viruses that do not have a lipid envelope. They are called picorna viruses. These viruses are very resistant to disinfectants. Therefore, the main methods of disinfection are mechanical cleaning (removal and combustion of hive remains) and additional use of oxidizing agents for disinfection of equipment. Burning and annealing help to disinfect the equipment. The causative agents of American bee brood plague and European bee brood plague are considered to be the most important bacteria that cause diseases of honeybees. Both diseases are highly contagious. Generally, these bacteria can be destroyed by high temperatures. A 5% solution of caustic soda acts against the spores of the American bee brood plague only if heated to 80 ° C. In a mixture with sodium hypochlorite, caustic soda is effective even at room temperature. Vegetative forms and fungal spores are not as resistant as bacteria, so disinfection against them is more successful. Fungi can be killed by the action of high temperatures and most of the above-mentioned chemical disinfectants (Plavša and Pavlović, 2017; Bojanić Rašović, 2018b, 2018c, 2018d; Bojanić Rašović, 2020a, 2020b; Ohashi *et al.* 2020; Nedialkov and Bižev, 1986; Rašić, 2018; Anon, 2020).

CONCLUSIONS

Disinfection is a very important measure that is implemented in order to prevent and suppress infectious diseases of bees and contamination of honey and other bee products with harmful microorganisms. The disinfection procedure is very complex, and its efficiency depends on a large number of factors. Due to the toxicity and other harmful effects of disinfectants, and often their insufficient efficiency, physical methods of disinfection should be applied whenever possible (application of high temperatures: dry heat - burning, annealing, ironing, tanning; moist heat (cooking, treatment with heated water steam under pressure), UV radiation). The chemical disinfectants that give the best results in beekeeping are chlorine preparations (sodium hypochlorite), caustic soda, alkaline salts (Na_2CO_3) and oxidizing agents (peracetic acid). Chemical disinfection is often not successful, especially when it comes to the causative agents of American plague litters, which form very resistant spores. In that case, the only way is to burn the hives, so that they are not a source of infection.

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Šeremešić, S., Marinković, D., Manojlović, M., Jovović, Z., Ćirić, V., Vasin, J., Vojnov, B. (2021): Soil organic matter pools and aggregate fractions in organic and conventional winter wheat cropping in Vojvodina province of Serbia. *Agriculture and Forestry*, 67 (3): 177-189

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SOIL ORGANIC MATTER POOLS AND AGGREGATE FRACTIONS IN ORGANIC AND CONVENTIONAL WINTER WHEAT CROPPING IN VOJVODINA PROVINCE OF SERBIA

SUMMARY

There is a lack of information on differences between organic and conventional soil management practices effects on the soil organic matter and aggregate fractions. Therefore, our research aimed to investigate those land-use systems to better understand the relationship between soil organic carbon (SOC) and soil structural properties in field crops production. For the purpose of this study, six locations under winter wheat, representing Haplic Chernozem soil type, were surveyed at 0-30 cm depth. On average, the distribution of soil aggregate fractions and soil organic matter (SOM) content was comparable in organic and conventional farms. Higher content of total and labile SOC was obtained from >2000 μm fraction being most important fraction in the turnover of soil organic matter. The degree of C saturation turned out to be an important regulator of SOC stability and turnover rates while carbon sequestration rate (2.64-2.84 kg m^{-2}) indicated the high potential of C increase in Chernozem soil. Detection of soil quality improvement in organic farming systems requires a longer period of time due to changes in utilization and a management practices. Direct links between labile carbon and soil physical condition are being pursued to help organic farmers manage soil resources more efficiently.

Keywords: land-use systems, fraction, total and label SOC

INTRODUCTION

Increasing demand for healthy food in the Republic of Serbia led to the conversion of conventional farms to organic, which could help facilitate soil

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quality preservation and environmental protection in agriculture. Properly designed tillage methods, fertilization, selection of varieties and balanced plant and animal production can exert significant effects on over all sustainability of the farms that can be a pillar in redesigning food systems to maximize ecological, economic, and social co-benefits (Kovačević and Dolijanović, 2017). Winter cereals under certified organic agriculture occupy the largest area, therefore, improving their production method could have a significant impact on the organic farming expansion (Šeremešić *et al.*, 2019). So far, more attention in organic production was given to the yield and economic performance of crops and less to soil improvement. As a result, in the agroecological conditions of Vojvodina, there is not sufficient research on the impact of sustainable land-use systems on soil OM changes. Therefore, soil resilience and protection in the organic plant production remains a challenge.

Given that organic cropping is intended to enhance soil performance by adjusting the relationship of soil organic matter (SOM) and soil physical properties, long-term research should be performed to better understand this process. According to Bronick and Lal (2005) developments of soil structure depends on the soil aggregation processes and mediates many physical soil functions and gives us the understanding of the carbon turnover and distribution in relation to applied management practices. At the same time, the level of total OM changes relatively slowly and the negative consequences on yield become noticeable only when the level of OM falls below 2% (Oldfield *et al.*, 2019), which can result in an irreversible process of degradation of all soil properties and complete loss of productivity. However, total OM pool is not sufficiently precise in monitoring changes caused by land use because of slower turnover rate. Therefore, quantification of the labile SOM fraction could be better indicator because it is directly controlled by management practices. Since the SOM is a major pool of soil organic carbon (SOC), sensitive to changes in climate or local environment (Schmidt *et al.*, 2011), investigation of fractions that is most prone to land-use changes are required (Ćirić, 2016). Labile SOC, such as hot water extractable organic carbon (HWOC) can be used as sensitive indicators of SOC change (Ghani *et al.*, 2003), as well as indicators of anthropogenic impacts to ecosystems (Ćirić, 2016). Fractions characterized as HWOC are free, easily mineralizing organic substances that pass through a 0.45 μm filter and consist mainly of carbohydrates derived from plant roots, microorganisms, amino acids, humic substances and to a lesser extent monomers of phenol and lignin, proteins and chitin (Leinweber *et al.*, 1995). The high level of biodegradability of the HWOC fraction affects soil CO_2 (Kim *et al.* 2012), as a result of which this fraction has a significant impact on the global carbon cycle and the effects of climate change. The HWOC fraction is the most active SOM compound, particularly sensitive to management practices and closely involved in aggregation and aggregate stability.

Since management strategy in organic agriculture relies upon fostering natural processes in soil, we anticipate that the labile SOM pool is a valuable

indicator of favorable management. It is well known that soil organic matter declines with tillage, insufficient fertilization, and removal or burning of crop residues (Šeremešić *et al.*, 2013). Also, it is considered that the loss of organic matter (OM) in the soil is one of the most pronounced consequences of intensive agricultural production (Bai *et al.*, 2018). On the other hand, modern understandings of the role of soil in sustainable agriculture start from the harmonization of production with natural fertility and soil quality (Ćirić *et al.*, 2014). Therefore, since the persistence of soil organic carbon is primarily an ecosystem property (Schmidt *et al.*, 2011), this study aimed to access the effects of these two management practices on SOM stabilization and aggregate fraction distribution. We expect, as soil management practices differ in these two types of systems, that organic farming systems can preserve the SOM pool in all of its fractions. Nevertheless, there is no universal solution to the selection of management practices that favor soil structure stabilization and organic matter preservation. Though, an individual approach is required for each farm to assure proper selection of agro-biological measures suitable for pedo-climatic conditions. Therefore, the aim of this study was to investigate differences between organic and conventional land-use systems to better understand the relationship between soil organic carbon (SOC) and structural properties in field crops production.

MATERIAL AND METHODS

Our study encompasses six different locations in Vojvodina Province of Serbia on in a temperate climatic condition. Soil samples were obtained from organic and conventional land use systems from the plots where winter wheat was grown. The criteria for the organic farms were that at least 5 years they have been certified as fully organic production. Sampling was performed in April and May, simultaneously at organic and conventional systems before winter wheat harvest. Three composite soil samples per plot, representing each system, were taken with a soil auger from the topsoil layer (0-30 cm) from the booting to the flowering stage of winter wheat. On the same plots, the disturbed soil samples (0.5 kg) were collected, gently manually broken, transferred to carton boxes, and stored as air-dried samples at room temperature prior to analysis of soil structure. Soil texture was determined using the pipette method, with sodium pyrophosphate as a dispersing agent. Aggregate fraction distribution was determined by the standard dry-sieving method (Savinov, 1936). Briefly, 500 g of air-dried, undisturbed sample is sieved through a nest of sieves having 10, 5, 3, 2-, 1-, 0.5-, and 0.25-mm square openings so eight aggregate size classes are obtained (>10, 10-5, 5-3, 3-2, 2-1, 1-0.5, 0.5-0.25 and <0.25 mm).

Aggregate size distribution, expressed as the structure coefficient (Ks), is calculated according to Shein *et al.* (2001) by using the formula:

$$K_s = a / b$$

where a represents the weight percentage of aggregates 0.25-10 mm and b represents the weight percentage of aggregates <0.25 mm and >10 mm (Ćirić *et*

al., 2012). Soil structure was also determined by wet sieving method procedure to obtain 4 classes of aggregates >2000 μm , 250–2000 μm , 53–250 μm and <53 μm . The indicator used to determine the stability of soil structural aggregates is the mean weight diameter (MWD), according to (Hillel, 2004):

$$\text{MWD} = \sum_{i=1}^n \bar{x}_i w_i$$

Percentage of waterstable aggregates (% WSA) was calculated as follow

$$\text{WSA (\% soil >250}\mu\text{m)} = \frac{\text{WSA-S}}{(\text{Wag} \times \text{K}) - \text{S}} \times 100$$

Where WSA represents the mass of waterproof soil aggregates after drying in the dryer, Wag - total weight of soil sample (100g), S - mass fraction of coarse sand and K – correction factor (total mass of all fractions after drying / mass of air-dry soil). SOC in total soil samples was determined by using the dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) wet oxidation method with external heating, followed by titration with ferrous ammonium sulfate (Mohr's salt). Grinding the samples in a mill and sieving through a sieve with 2 mm diameter preceded the analysis Labile carbon, considered the portion of SOC, was extracted with hot water (HWOC) using the Ghani *et al.* (2003) procedure. 10 g of the soil of each 4 classes of aggregates were put in a 50 ml cuvette (< 2 mm) and 40 ml of distilled water was added to the air-dried soil. The cuvettes were put into a horizontal shaker on 30 rpm for a period of 30 min. After that, the samples in the cuvettes were transferred into a steam hot-water bath at 80 °C temperature, for a period of 16h. The next phase involved centrifugation on an MSEc centrifuge (Measuring & Scientific Equipment LTD., London) with 3000 rpm for 20 minutes. After centrifugation, the substrate was filtered through a 0.45 μm ME 25/21 CT filter. Determination of labile carbon content, in each soil class of aggregates, was done by the Tyrin's titrimetric method using dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) with external heating, followed by titration with ferrous ammonium sulfate (Mohr's salt). To calculate the potential of C saturation soil particles <20 μm was done with equation of Hassink (1997) $C_{\text{satpot}} = 4.09 \times 0.37 \times \leq \mu\text{m particles (\%)}$, where C_{satpot} is potential of C saturation (mg g^{-1}) of fine soil particles. The difference between the protection capacity of the fine fraction and current C content correspond to saturation deficit $C_{\text{satdef}} = C_{\text{satpot}} - C_{\text{cur}}$ where C_{cur} represent the current mean of C in fine fractions. C_{cur} in fine fraction was calculated with the formula $C (\text{g kg}^{-1}) = (\text{HWOC} (\mu\text{g g}^{-1})) - 134,3/21,54$ ($r=0,87^{**}$) (Šeremešić *et al.*, 2013). The total amount of the C sequestration potential was calculated using the Wiesmeier *et al.* (2014) equation $C_{\text{seq}} = C_{\text{satdef}} \times \text{bulk density} \times \text{depth} \times 10^{-2}$. The obtained data were analyzed statistically by analysis of variance (ANOVA) using the software system STATISTICA 12.6, StatSoft, Inc.

RESULTS AND DISCUSSION

Comparison of different land-use systems reveals similar chemical properties between organic and conventional land use systems (Table 1). Based on the results of agrochemical analyzes of soil samples taken from a depth of 0-

30 cm, all sites were neutral in pH reaction. SOM content in the bulk soil samples largely varies from 1.79 to 3.94%, but the average content of SOM is similar in organic and conventional plots 3.09% and 3.08%, respectively. Given that the soils in Vojvodina province are under pressure of SOM loss (Šeremešić *et al.*, 2020), the obtained results reveals that the selected locations are representing majority of arable soils. In addition to that, based on SOM values, selected locations for organic agriculture are partially suitable for this particular system of agriculture. The most fertile soil is located at the Nadalj and the lowest sampling location was Šuljam at Fruška Gora Mountain. Comparative analyses of Harchegani-Kiani *et al.* (2019) also indicated that the different land use type has not display significant effects on SOC in calcareous soils of semi-arid climate.

Table 1. Chemical properties of soil samples of different land-use systems

Location	System	pH		CaCO ₃ %	SOM %	Total N %	AL-P ₂ O ₅ mg/100g	AL-K ₂ O mg/100g
		KCl	H ₂ O					
<u>Pančevo</u>	org	7.10	7.86	8.70	3.94	0.20	25.78	19.73
	con	7.26	8.04	10.77	3.53	0.18	16.68	18.97
<u>Šuljam</u>	org	6.89	7.97	3.31	1.93	0.10	5.30	17.99
	con	7.06	8.02	3.73	1.79	0.09	8.71	18.36
<u>Nadalj</u>	org	7.11	7.86	4.14	3.79	0.19	21.38	20.54
	con	7.10	7.97	4.14	3.84	0.19	9.59	19.21
<u>Temerin</u>	org	6.94	7.96	2.07	2.87	0.14	4.95	24.26
	con	6.42	7.31	2.49	3.29	0.16	5.67	31.34
<u>Zemun</u>	org	7.18	8.03	5.80	2.46	0.12	26.34	29.68
Polje	con	7.01	7.76	3.31	2.68	0.13	23.96	23.74
<u>Pivnice</u>	org	7.27	8.11	8.70	3.57	0.18	13.36	16.30
	con	7.21	8.00	6.22	3.37	0.22	11.74	24.99
Average	<u>Org</u>	7.08±0.14	7.96±0.10	5.45±2.7	3.09±0.8	0.16±0.04	16.19±9.7	21.42±4.8
	<u>Con</u>	7.00±0.30	7.85±0.12	5.11±3.4	3.08±0.7	0.16±0.05	12.73±6.61	22.78±5.2

The mechanical composition reveals that soil belongs to loamy clay with favorable ratio of clay and loamy fractions. Based on the analysis of the mechanical composition, soil samples are significantly uniform by localities, but also by individual comparisons (Table 2). In this way, preconditions are created for comparison of soil structural and analyses of soil organic matter in order to interpret the impact of the production system on other physical and chemical properties of the soil. Greater variation was found in the content of clay and fine sand compared to silt fraction. Vojvodinian Chernozem is considered to be a well-structured soil. Our study confirmed this hypothesis. This can be explained

with the facts that at the time of sampling winter wheat crop stand protected soil from disintegration processes and dispersion.

Table 2. Soil texture of soil samples from a different location and land-use systems

Location	Parcel	Coarse sand	Fine sand	Silt	Clay	Total sand	Silt + Clay	Texture class (Tommerup)
Pančevo	org	0.70	46.66	26.56	26.08	47.36	52.64	loamy clay
	con	0.90	46.26	29.28	23.56	47.16	52.84	clay loam
Šuljam	org	0.20	36.84	32.24	30.72	37.04	62.96	loamy clay
	con	0.30	39.46	30.64	29.60	39.76	60.24	loamy clay
Nadalj	org	0.10	43.22	30.08	26.60	43.32	56.68	loamy clay
	con	0.90	45.02	27.84	26.24	45.92	54.08	loamy clay
Temerin	org	1.10	39.42	24.08	35.40	40.52	59.48	loamy clay
	con	0.60	37.52	26.08	37.80	34.12	63.88	loamy clay
Zemun	org	0.30	44.10	28.12	27.48	44.40	55.60	loamy clay
Polje	con	0.60	40.96	30.28	28.16	41.56	58.44	loamy clay
Pivnice	org	1.00	42.00	30.64	26.36	43.00	57.00	loamy clay
	con	1.00	45.72	28.96	24.32	46.72	53.28	clay loam
	Org	0.57	42.04	28.62	28.77	42.61	57.39	loamy clay
	Con	0.72	41.82	29.18	28.28	42.54	57.46	loamy clay

The results of dry sieving show that soils from organic plots have the largest representation of the aggregates in size of 10-5 mm, whereas the lowest representation of the aggregate of <0.25 mm. Samples from the conventional plots have the largest representation of the aggregate size 2-1 mm and were lowest in <0,25 mm and 0,5-0,25 mm aggregate size (Table 3). However, comparing organic and conventional land-use systems after dry sieving reveals a differences in the fractions of 0.5 - <0.25 mm in favor of conventional agriculture indicating less dispersed soil that can better resist the occurrence of the water and eolian erosion. Consequently, this could be attributed to the favorable crop density and root traits can contribute to the erosion control and maintenance of soil physical properties (Le Bissonnais *et al.*, 2018). On the other hand, a higher proportion of the 10-5 mm aggregates under organic systems indicated poor soil preparation and potential compaction. In average, in this study we determined

82,4% agronomically valuable aggregates (10-0.25 mm size). Our results are comparable with findings of Ćirić *et al.* (2012) on Chernozem in which 84.09% of aggregates after dry sieving was classified as agronomically valuable. Commonly, organic farmers are less equipped with the efficient machinery compared to conventional farmers. Based on the obtained results of soil structural properties, we speculate that plots in organic production rely on shallow, or conservation tillage which is not sufficient for crop requirement that aggravates weed control (Nikolić *et al.*, 2018; Rajković *et al.*, 2021). It can be noticed that the production system does not affect much the structure coefficient (K), with the exception of the Nadalj location, where the structure coefficient is significantly higher in the soil samples from the organic plot. This can be explained by the fact that soil in Nadalj has the highest content of organic matter and has been regularly receiving the animal manure. For both land-use systems, wet sieving showed the least distribution of higher aggregate fraction $>2000 \mu\text{m}$ in the 0-30 cm layer, which is in line with previous research (Šeremešić *et al.*, 2020). The highest representation of aggregates was in the fractions 250-53 μm and $<53 \mu\text{m}$ (Table 3). It should be emphasized that the distribution of large fractions is conditioned by the soil tillage, while for the smaller fractions the effect of the quality and quantity of organic cementations substances is more important and independent of tillage (Zhang *et al.*, 2017). Jugović *et al.* (2020) showed that selection of an optimal system of soil tillage can decrease the soil degradation for the purpose of the environment protection. Soil samples after wet sieving procedure showed that there are more aggregates of 250-53 μm and $<53 \mu\text{m}$ size on organic plots compared to conventional plots. Conversely, on conventional plots, the aggregates $>2000 \mu\text{m}$ and 2000-250 μm size were highest (Table 3). It follows that the soil from plots in organic production can be less resistant to rainfall dispersion. Our findings are contrary to those reported in Nešić *et al.* (2014) study in which the stability of soil aggregates was higher in the soils at the organic farms across different soil types. Based on the average MWD and WSA, we can assume that the individual methods used for tillage had a significant impact on these indicators to a greater extent compared to the land-use systems. Stabilization of the physical properties of soil in organic production requires a longer period of time and adaptation of the different tillage methods. Given that the average structural properties of two land use systems are similar at each location individual approach must be chosen to improve cropping management toward SOC and structure preservation.

Soil chemical properties of investigated sites reveals that the average total SOM content of different land use system (Table 1.) were similar and belongs to the soil class well provided with humus, such as more than 50% of arable land in AP Vojvodina (Sekulić *et al.*, 2010). Average values from conventional and organic plots indicate that the content of labile organic matter is lower on organic plots (Table 5). High WEOC extractability indicates the presence of potentially readily degradable SOC, whereas low extractability indicates the presence of SOC with low degradability (Breulmann, 2011).

Table 3. Soil dry aggregate size distribution and size classes of stable aggregates of wet sieving

Location	System	Soil dry aggregate size distribution										Wet sieving				
		Aggregate class %										Size classes of stable aggregates (μm)				
		>10	10-5	5-3	3-2	2-1	1-0.5	0.5-0.25	<0.25	8000-2000	2000-250	250-53	<53	MWD (mm)	WSA (%)	
Pantčevo	org	5.29	12.70	11.12	8.18	14.26	16.78	15.80	15.84	3.73	4.83	20.30	40.04	35.35	0.53	25.00
	con	5.56	16.03	14.02	8.53	14.31	15.35	12.33	13.87	4.15	2.30	27.96	43.80	25.94	0.50	30.26
Šuljani	org	22.45	31.16	14.17	8.46	12.01	7.19	2.92	1.63	3.15	7.32	20.09	28.07	44.51	0.53	27.00
	con	19.92	26.07	13.31	9.49	15.64	9.98	3.72	1.86	3.59	6.30	24.00	27.70	44.04	0.63	30.00
Nacali	org	4.06	15.45	15.06	10.18	20.72	20.04	10.34	4.54	10.67	2.26	17.22	38.80	41.71	0.37	19.00
	con	5.64	9.98	14.40	12.83	25.74	18.16	7.96	5.28	8.17	11.53	26.85	26.40	35.21	0.91	38.38
Temerin	org	15.51	32.08	17.00	10.49	13.56	6.87	2.99	1.48	4.88	11.90	34.26	24.78	28.75	1.02	46.00
	con	16.98	17.84	13.36	10.30	19.69	14.20	5.77	1.85	4.31	5.72	33.93	26.31	34.05	0.71	39.65
Zemun	org	11.50	19.70	15.03	10.04	17.99	16.09	7.23	2.42	6.18	2.02	19.68	29.19	49.09	0.38	22.00
Polje	con	12.18	16.83	15.26	10.85	20.43	16.04	6.60	1.80	6.15	3.35	25.83	36.80	34.01	0.52	29.00
Pivnice	org	15.47	13.49	11.06	8.37	17.45	16.96	11.40	5.79	3.70	4.55	18.42	38.01	39.01	0.61	23.00
	con	16.73	16.20	13.11	10.30	17.23	14.28	8.62	3.62	3.92	4.49	24.58	32.68	38.25	0.55	29.00
	org	12.38±6.9	20.76±8.7	13.91±2.3	9.29±1.0	16.00±3.2	13.99±5.5	8.45±5.0	5.28±5.4	5.39a	5.48±3.6	21.66±6.8	33.15±6.5	39.74±7.1	0.57a	27.00b
	con	12.84±6.1	17.16±5.9	13.91±0.8	10.38±1.4	18.84±4.1	14.67±2.7	7.50±2.9	4.71±4.6	5.05a	5.62±3.2	27.19±3.6	32.28±6.9	35.25±5.9	0.64a	32.72a

Ks-; MWD; WSA; **Numbers marked with the different color are significant at $p<0.05$ level

The obtained results can be interpreted by the application of mineral fertilizers on conventional plots, i.e., the absence of adequate fertilization in organic production. According to Huang *et al.* (2020) N addition increased soil C stocks and decrease decomposition of old soil C. In organic wheat production, foliar fertilizers are used, which are not sufficient to activate the soil and create satisfactory amounts of assimilates. Generally, > 2000 μm fractions had the highest content of HWOC, while decreasing the size of the fractions reduces its HWOC content, except for fraction <53 μm , in the organic system. Comparing the content of labile organic matter by fractions and by type of production shows that fractions >2000 μm , and 250-53 μm from conventional plots contain more labile organic matter compared to the same fractions from organic production, while in fractions 2000-250 μm and <53 μm , higher content of labile organic matter was determined in samples taken from organic plots. Given the carbon content in the different aggregate fractions, obtained data support the aggregate hierarchy model. Soils under organic agricultural system manage to preserve the same amount of the SOC in the condition with organic fertilization and potentially lower input of fresh C from the crop residue and less mineral nitrogen. These results are different from those presented by Marriott and Wander (2006) where organic farming increased the SOC by 14% compared with conventional counterparts probably because of a variety of soil types used in this study. In this sense, preservation derives from the manure application, also elaborated in Spiegel *et al.* (2010). Stabilization of the physical properties of soil in organic production requires a longer period and adaptation of the tillage system.

However, in addition to the carbon content in the soil, the dynamics of carbon is much more important, which indicates the connection between organic matter and soil structure (physical soil). Hassink (1997) found a strong correlation of SOC stored in the fraction containing silt and clay particles in a wide range of topsoils of temperate and tropical regions that can be a basis of assessment for stable SOC saturation. The saturation deficit C_{satdef} in our study point to the amount of carbon that can be stored in soil until the protection capacity is reached (Table 6). C_{satdef} was higher at organic plots compared to conventional one possibly as consequence of reduced mineral nitrogen application that is required for balanced C/N ration that lead to C sequestration. Conversely, somewhat higher C_{seq} was calculated at organic systems (2.84 kg m^{-2}) compared to conventional (2.64 kg m^{-2}). In his study Wiesmeier *et al.* (2014) showed higher sequestration potential of cropland ($>4 \text{ kg m}^{-2}$) that correspond to our findings. Enhancing the SOM pool is a major challenge in both land-use systems, but benefits have long-term effects since include improvement in soil structure, retention of water and plant nutrients, increase in soil biodiversity and decrease in risks of soil erosion and the related degradation (Lal, 2009).

C_{satpot} ration in C bulk soil indicate the overall (historical) loss of soil organic carbon in soil. We found that for Chernozem soil this value is amounted to 71-72% which leads to the conclusion that in long-term soil has lost approximately 30% of initial soil organic carbon in the topsoil. This could be

mainly attributed to tillage but also to other unfavorable agronomic practices such as residue burning.

Table 5. HWOC content in water-stable aggregates

Location	System	HWOC ($\mu\text{g g}^{-1}$)				Average
		Size classes of stable aggregates				
		8000-2000	2000-250	250-53	<53	
Pančevo	org	425.3	329.8	271.4	254.3	332.7
	con	451.2	386.8	298.7	255.3	335.5
Šuljam	org	305.1	271.7	184.4	222.2	245.8
	con	369.8	213.9	235.8	170.5	247.5
Nadalj	org	429.2	362.9	242.1	257.7	322.9
	con	332.3	328.9	353.7	235.8	312.7
Temerin	org	365.5	241.7	246.5	253.3	289.1
	con	373.5	295.7	212.4	194.8	256.5
Zemun	org	383.7	280.9	154.2	166.9	246.4
Polje	con	366.5	248.5	195.3	177.1	246.8
Pivnice	org	371.2	283.9	236.9	212.4	276.1
	con	374.9	338.7	255.0	231.9	300.1
Average	org	388.3±64	295.1±48	230.8±59	227.8±38	285.5
	con	378.1±43	285.3±55	258.5±65	210.8±37	383.1

Table 6. Carbon sequestration potential of investigated land use systems

Cropping system	C_{satpot} (g kg^{-1})	C_{cur} (g kg^{-1}) (fine fraction)	C_{satdef} (g kg^{-1})	C_{seq} (kg m^{-2})	C bulk soil (g kg^{-1})	$C_{\text{satpot}}/$ C bulk soil
Organic	25.33 ±1.3	15.06±3.9	10.27±4.3	2.84±1.2	17.92±4.6	71.5%
Conventional	25.07±1.6	15.56±4.1	9.51±5.5	2.64±1.5	17.88±4.2	72.2%

CONCLUSIONS

Our results showed similar content of SOM on both land-use systems indicating the capability of organic farms to preserve SOM with fewer inputs. We found higher content of soil organic matter stored in the micro aggregate size classes $>2000 \mu\text{m}$ indicating their importance in SOM preservation. Carbon saturation deficit is somewhat higher at organic plots as well as carbon sequestration rate. Accordingly, proper interaction of soil structural properties and soil organic matter fractions could assure sustainable development of organic

farms. It follows that the organic producers should pay attention on cultivation methods, timing of tillage operation and fertilization. Additional research is needed to obtain the quantitative basis for evaluating single amendment and practice to increase SOM content and macro aggregation as well as long-term soil fertility.

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