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DYNAMICS OF BIOMASS STOCKS IN AREAS OF SECONDARY FOREST LOCATED IN WESTERN AMAZON BETWEEN 2016 AND 2020

SUMMARY

Secondary forests are portions of forest areas that were previously deforested. In general, secondary forests are characterized by fast-growing species, an alternative for reducing net carbon emissions and mitigating climate change; However, these forest environments are still poorly studied. In this context, this work aimed to adjust predictive models of biomass concerning the height and diameter of trees, analyzing temporally and quantitatively the biomass stocks in areas of secondary vegetation located in Rondônia, Western Amazon, aiming at quantification of aerial biomass in these ecosystems and discuss the implication of abundance of individuals in the estimates. To adjust the allometric equations it was necessary to measure all trees with a circumference at breast height above 15 cm in plots with 200m². After the measurements, a plot tree representing the average diametric variable was selected and slaughtered to calculate its biomass. The adjusted equations presented adjusted r² ranging from 0.49 to 0.57, root means square error (RMSE) from 247 to 296 kg, and residual standard error (Syx) from 49 to 53 kg. In 2016, the plots had an average of 41.47 $t.ha^{-1}$ of biomass, and 2020 recorded 81.66 $t.ha^{-1}$. In this sense, we observed a total increase of 96.92% between 2016 and 2020. Through biomass estimates it was possible to observe that secondary forests are a potentially significant biomass sink due to the rapid accumulation rates of this component. Therefore, biomass stocks were increased over the years of this study, demonstrating the capacity for biomass growth in forests undergoing restoration.

Keywords: Allometric equations; Forest recovery; Amazon biome; Temporal analysis; Tropical Rainforest.

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INTRODUCTION

Human expansion in the Amazon biome has converted large areas of climax-stage tropical forest into landscapes consisting primarily of pasture, agriculture, and secondary forest mosaics. This type of forest is defined as those formed because of the human impact on areas with forest cover. According to Poorter et al. (2016) and Wang et al. (2020b), secondary forests comprise approximately 21 % of the previously deforested areas in the Brazilian Amazon. In general, secondary forests are characterized by fast-growing species (pioneers) and are good alternatives to reduce net carbon emissions, mitigating climate change (Chazdon et al. 2016).

In recent years, new forms of forest and ecological restoration that offer ways of converting degraded tropical forests have been tested. These restoration techniques include improvements in secondary forests management, reforestation, and enrichment planting as more complex reforestation where forest cover has been lost (Matos et al. 2019; Barros et al. 2020). While forest loss continues in Brazil at variable rates, techniques are implemented to develop secondary forests where primary forests have been completely removed by human processes.

The extent and age of Amazonian secondary forests have already been quantified, and their spatiotemporal patterns are highly dynamic (Wang et al. 2020a; Nunes et al. 2020). In this context, these forests are a crucial component in the Brazilian Amazon, as their cover restores the structure and nutrient cycling in the soil. Despite the importance of these forests for conservation planning, environmental policy, and management of forest areas in the Brazilian Amazon, they have been less studied compared to primary tropical forests (Barlow et al. 2007; Carvalho et al. 2019; Teixeira-Santos et al. 2020).

Understanding biomass stocks in secondary forests is essential for forest management and restoration; therefore, biomass estimates are highly sensitive to choosing a particular allometric equation (Chave et al. 2005; Van Breugel et al. 2011). Although local allometric models generally perform well for a particular location or forest type, they are inaccurate in biomass estimates when applied to other sites and different forest types (Chave et al. 2005; Sanquetta et al. 2014b, a; Feng et al. 2017; Corte et al. 2020; Tejada et al. 2020).

Estimating biomass at different scales mainly depends on equations or remote sensing techniques for prediction and analysis at regional and national scale mapping (Mohd Zaki and Abd Latif 2017; Mitchell et al. 2017; Tripathi et al. 2018). These equations are statistical models used to predict biomass based on tree measurement characteristics measured during forest inventory processes. Biomass estimation is particularly challenging in tropical forests due to difficulties with collecting field data in these ecosystems, characterized by a high heterogeneity of individuals, tree measurement variables, vertical structure, and horizontal distribution.

Allometric equations are important for their application in forest biomass and carbon assessments both locally and nationally. Generalized pan-tropical models of biomass estimation equations have been developed by several researchers (Kenzo et al. 2020; Virgulino-Júnior et al. 2020; Romero et al. 2020; Zhou et al. 2021; Latifah et al. 2021; Saha et al. 2021). The adjustments to these equations were obtained by measuring multiple tree species and several distinct locations and are intended to be applied to a wide variety of tropical forests. However, a large error is observed when estimates are generated by adopting generic pan-tropical allometric equations for specific forest types (Ngomanda et al. 2014; Vinh et al. 2019).

With limited research on secondary forests and their biomass stocks, it is possible that these stocks are quantified through allometric equations arising from measurements and field observations and that this prediction can be used for the temporal analysis of these stocks in forest environments. In this sense, this work aimed to adjust predictive models of biomass concerning the height and diameter of trees, analyzing temporally and quantitatively the biomass stocks in areas of secondary vegetation located in Rondônia, Western Amazon, aiming at quantification of aerial biomass in these ecosystems and discuss the implication of abundance of individuals in the estimates.

MATERIAL AND METHODS

This study was carried out in a region of the Amazon rainforest located in the state of Rondônia, Brazil, between the meridians 62º44'05" and 63º16'54" and parallels 9º00'00" and 9º30'00" of south latitude, as shown in Figure 1. The areas refer to forest restoration plantations, containing 20 plots with 200 m² (20 x 10 m) monitored annually to assess the growth of trees, natural regeneration of new species, and biomass stock monitoring (Figure 1).

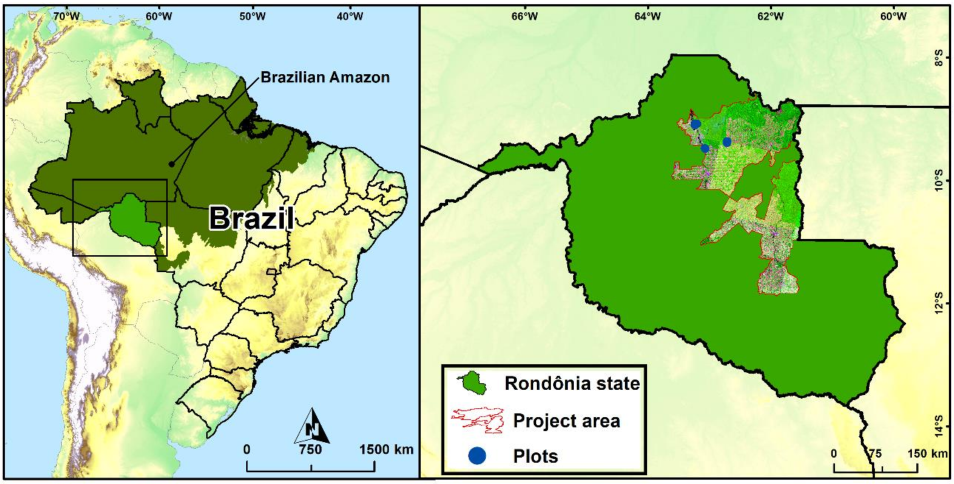

Figure 1. Location of the study area and experimental plots.

All trees in the plots with a circumference at breast height (CBH) above 15 cm were identified to measure their CBH and total height (ht), the circumferences were measured using tape measures and total heights were measured with telescopic rods. The species that could not be identified in the field had some branches collected and, when possible, also the reproductive structures. Exsiccates were made, identified, herborized, and registered at the Federal University of Rondônia (UNIR), with the support of the Museu Paraense Emilio Goeldi de Botânica.

Acquisition of forest biomass

After the forest inventory, the species with the greatest phytosociological importance were listed to determine the biomass by the destructive method. On the other hand, the diametric distribution was used to select individuals to be slaughtered and measured; that is, the average diameter at breast height (DBH) of the plot was represented by a single individual that had approximately that diameter.

To determine the biomass, 30 trees were felled and sectioned into bole, branches, foliage, and miscellaneous (fruits, flowers, shoots, among others). The root system was exposed until 50 cm depth; all exposed parts were collected and cleaned. The fractions collected from the trees were weighed separately using a digital scale with a precision of 100g, obtaining the fresh weight of each compartment. Approximately 500g biomass was taken from each fraction of fresh samples and placed in single packages to be taken to the laboratory. These samples were dried in a forced circulation oven at 65 ºC until reaching constant weight. Then, the conversion into dry biomass of each compartment was performed according to Equation 1.

$$bs_x = bf_x \frac{(100 - u_x)}{100} \qquad \textbf{eq. 1}$$

Where:
bs_x = tree dry biomass of fraction x (kg);
bf_x = tree fresh biomass of fraction x (kg); and
u_x = tree moisture content of fraction x (%).

To obtain the total biomass of each tree, the sum of each biomass fraction was performed according to Equation 2.

$$W = \sum ba + bf + br \qquad \textbf{eq. 2}$$

Where:
W = total biomass (kg);
ba = tree aboveground dry biomass (kg);
bf = tree bole dry biomass (kg);
br = tree root dry biomass (kg).

Adjusting the equations

The data obtained in the field - ht, Biomass (W), and CBH (later converted to DBH - diameter at breast height) - were used to adjust equations to estimate tree individual biomass. The R software version 4.04 (R team, 2021) was used to adjust the equations with the *olsrr* package from which by inserting the variables (dependent and independent), it is possible to obtain all combinations between the independent variables. In addition to linear equations, we also analyzed non-linear, exponential, logarithmic, and polynomial models, as shown in Table 1.

Table 1. Generic equations used in adjustments

Generic equations
$y = x_1 + \cdots + x_k$
$y = x_1 + \cdots + x_k + [\log(x_1 + \cdots + x_k)]$
$y = x_1^2 + \cdots + x_k^2$
$y = \log(x_1 + \cdots + x_k)$
$y = \dfrac{x_1 + \cdots + x_k}{x_2 + \cdots + x_k}$
$\log y = x_1 + \cdots + x_k$
$\log y = x_1^2 + \cdots + x_k^2$
$\log y = x_1^2 + \cdots + x_k^2 + [\log(x_1 + \cdots + x_k)]$
$y = x_1^{x_2} + \cdots + x_k^{x_j}$
$y = x_1^{\beta_1} + \cdots + x_k^{\beta_j}$
$y = \exp(x_1 + \cdots + x_k)$
$y = \exp(x_1^2 + \cdots + x_k^2)$

For all models tested in this study, biomass (kg) was used as dependent variable (y), and DBH and ht as independent variables. The Kolmogorov-Smirnov normality test was used to check the residuals normality of the dependent variable with the independent. The Bartlett variance homogeneity test was used to compare the variance of two or more samples to decide whether they are taken from populations with equal variance.

The selection of the model that best suited our data was performed using adjustment metrics: sum of squared estimate of errors (SSE), r^2 (determination coefficient), adjusted r^2 (adjusted determination coefficient), RMSE (root mean squared error), Bias, x^2 (chi-square), Syx% (standard error of the estimated percentage) and Syx (estimated standard error). The Meyer Correction Factor was used to correct the logarithmic discrepancy in models in which the dependent variable was submitted to logarithmic transformation

Model validation was performed using the K-fold technique with separation of 10 subsets (k = 10); this cross-validation method involves dividing the dataset into k-subsets. Each subset is maintained while the model is trained on all other subsets; the fitted model is then used to test the subset that was not used. The models that obtained the best statistical metrics were submitted to visual analysis of their fit lines (Observed data versus Adjusted data) and analysis of standardized residuals.

RESULTS

Table 2 shows the characteristics of the 30 trees cut in sections and measured. These individuals represented 22 species and 11 families (Table 2). Diameters ranged from 5.41 to 13.06 cm, with a mean of 9.47 cm and a standard deviation of 2.57 cm. Heights ranged from 5.23 to 11.80 m, with a mean of 8.18 m and a standard deviation of 1.75 m.

Table 2. Species collected and descriptive statistics of diameters and heights of felled trees for biomass calculation.

Family	Epithet	DBH (cm)	*ht* (m)
Anacardiaceae	*Anacardium* sp.	9.87	8.90
Bignoniaceae	*Handroanthus* sp.	12.10	8.00
	Handroanthus sp.	12.29	8.46
	Handroanthus serratifolius L.	10.83	7.02
Bixaceae	*Bixa orellana* L.	7.17	7.00
Boraginaceae	*Cordia alliodora* (Ruiz & Pav.) Cham.	12.19	10.97
Euphorbiaceae	*Hevea brasiliensis* L.	8.12	9.35
Fabaceae	*Stryphnodendron* sp.	10.50	8.80
	Hymenolobium pulcherrimum Ducke	6.05	6.15
	Inga cylindrica (Vell.) Mart.	10.98	8.60
	Enterolobium sp.	5.89	5.80
	Schizolobium amazonicum Huber ex Ducke.	12.73	10.80
	Parkia multijuga Benth.	11.94	7.70
	Hymenaea courbaril L.	13.05	9.80
	Enterolobium schomburgkii (Benth.) Benth.	5.73	6.30
	Schizolobium amazonicum Huber ex Ducke.	7.42	7.35
	Hymenaea courbaril L.	5.44	5.23
	Apuleia leiocarpa (Vogel) J. F. Macbr.	9.90	9.50
	Dipteryx odorata (Aubl.) Willd.	7.48	6.55
	Enterolobium schomburgkii (Benth.) Benth.	9.24	9.90
	Dipteryx odorata (Aubl.) Willd.	7.32	11.00
	Enterolobium schomburgkii (Benth.) Benth.	9.39	8.60
	Acacia mangium Wild.	13.06	9.20
	Parkia multijuga Benth.	7.64	6.95
Hypericaceae	*Vismia guianensis* (Aubl.) Choisy	5.41	7.00
Malvaceae	*Ceiba pentandra* (L.) Gaertn.	10.66	6.25
Melastomataceae	*Bellucia grossularioides* (L.) Triana	8.92	6.40
Meliaceae	*Cedrela odorata* L.	7.51	6.65
	Cedrela odorata L.	13.06	9.50
	Descriptive statistics	**DBH (cm)**	***ht* (m)**
	Average	9.47	8.18
	Standard error	0.47	0.32
	Median	9.63	8.23
	Standard deviation	2.57	1.75
	Sample variance	6.61	3.07
	Minimum	5.41	5.23
	Maximum	13.06	11.80

Figure 2 shows the diametric distributions for 2016 and 2020. In the first year of monitoring, there were more trees with smaller diameters than those measured in 2020, when fewer individuals with larger diameters were measured. In this sense, this image shows that the years of monitoring have seen a mortality of trees and a development of the remaining trees. The blue column shows the individuals measured in 2016 and the yellow column shows the individuals in 2020, it can be seen that there is a higher frequency of trees in 2016 compared to 2020.

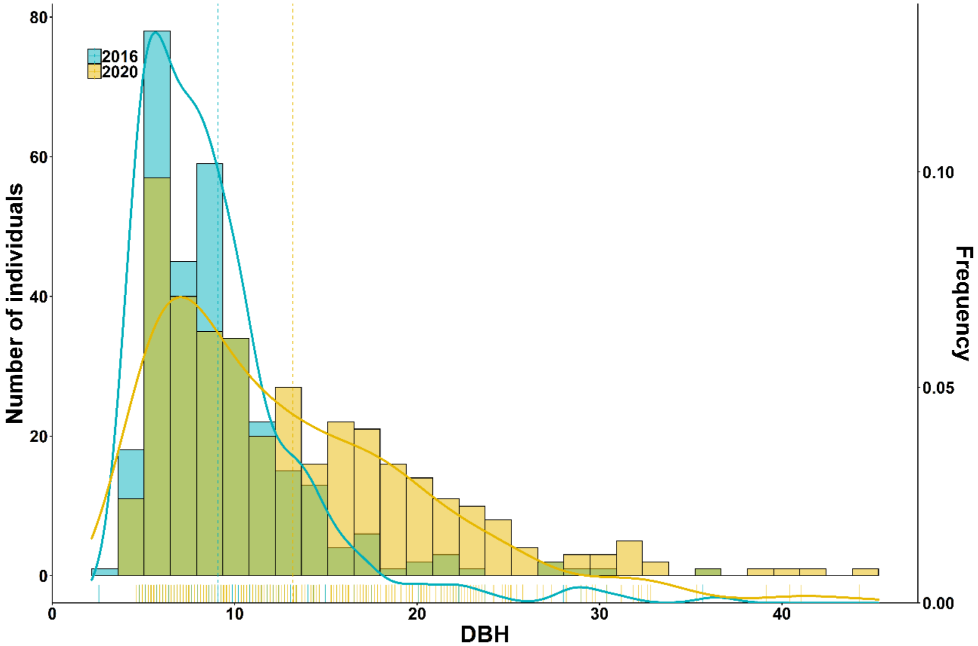

Figure 2. Diametric distributions of trees in 2016 and 2020.

Adjusted equations

The Kolmogorov-Smirnov test showed that the residuals are normal (ρ = 0.2514). Thus, it was possible to adjust the models without transforming the variables. We also obtained the homogeneity of the variance through the Bartlett test (ρ = 0.4304).

Figure 3 demonstrates the comparison of observed and adjusted data from the five best models. It presented similar behaviors to the adjustment, with an overestimation when the biomass exceeds 35 kg. The residuals demonstrate that this overestimate can reach up to 25 kg more than the observed biomass and, with values less than 35 kg, there is an underestimate varying up to 25 kg less.

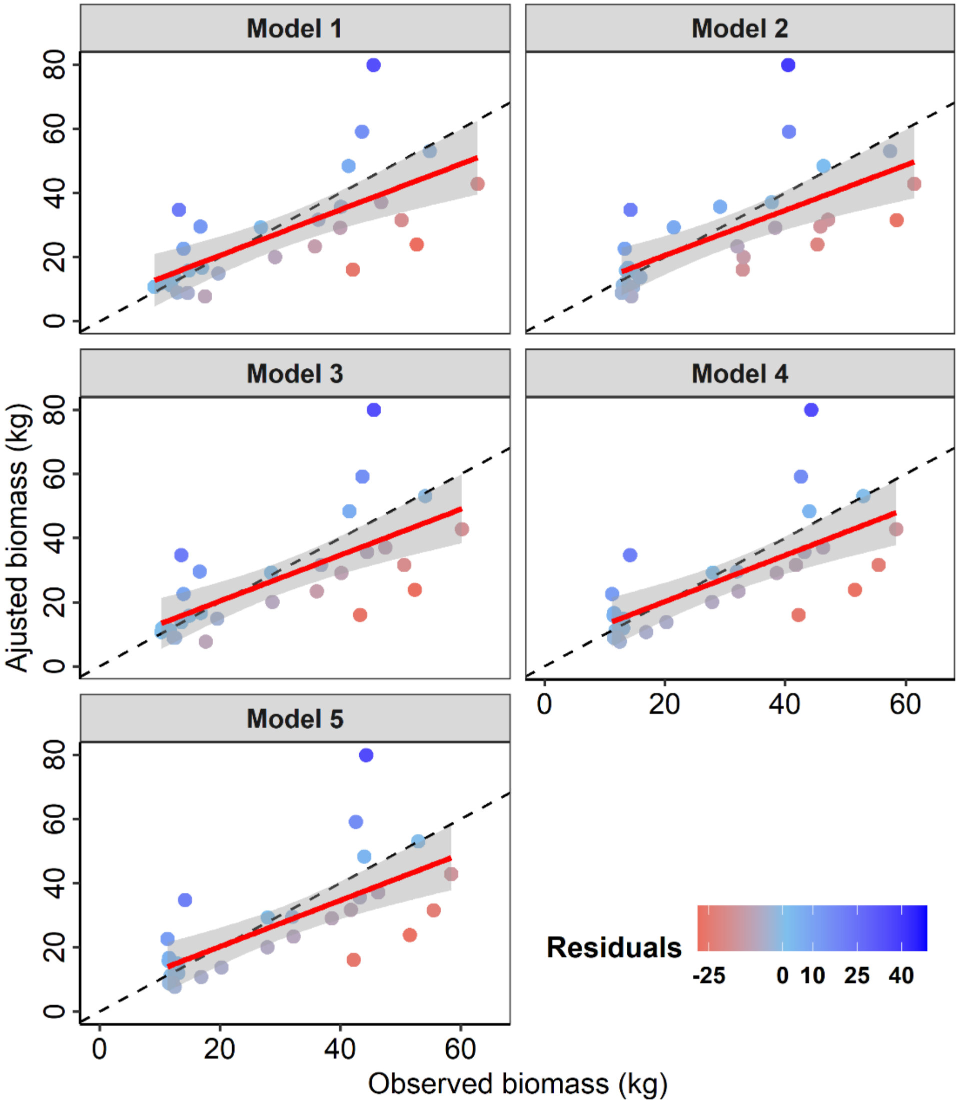

Figure 3. *Comparison* of the five best models against observed and adjusted data.

Table 3 shows the five best fits for predicting biomass using the DBH and total height variables. Model 1 presented the best values concerning comparative statistics. Later, this model was used to calculate the biomass for all trees measured in the plots for each year.

The residuals shown in Figure 4 demonstrate that the error variances are constant (homoscedastic) and that the independent variables (DBH and ht) have a linear relationship with the dependent variable (w). We observed that the standardized residuals were randomly dispersed around zero, with constant variance, concentrated between -1 and 1.

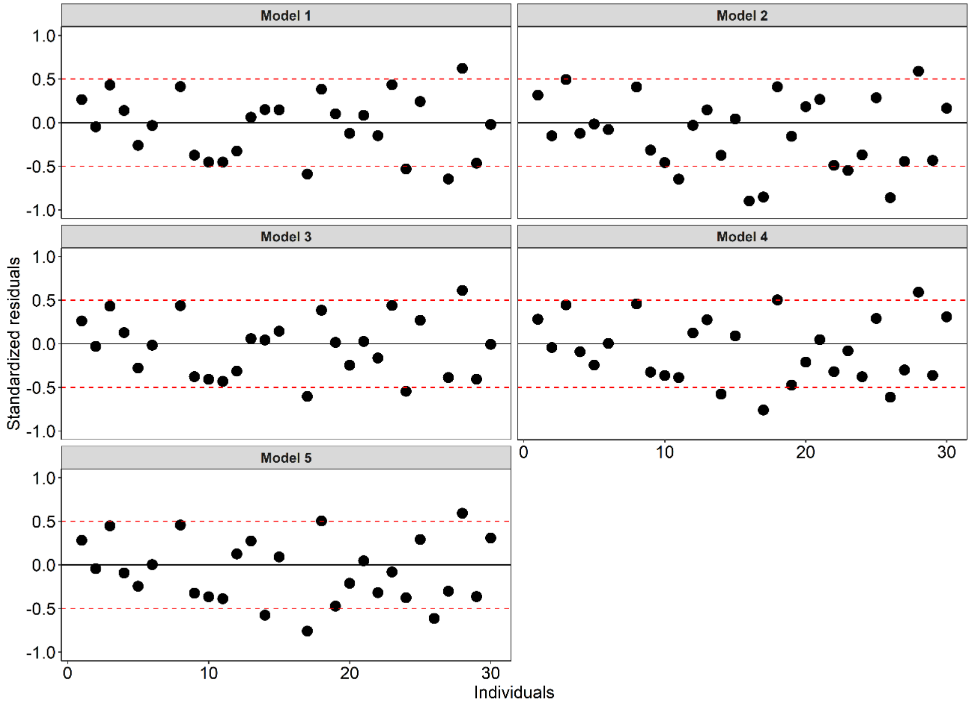

Figure 4. Graphical distribution of total biomass standardized residuals for the five best-fitted models, the y axis indicates the standardized residuals, while the x indicates the total observations.

Table 3. Five best equations obtained through adjustments, according to statistical criteria.

	Models	SSE	r^2	r^2_{ajus}	Bias	RMSE	Syx (kg)	Syx %	x^2
1	$w = 142.54 - 35.25DBH - 18.08ht + 5.77DBH.ht$	7416.86	0.55	0.57	-9.28×10^{-15}	247.22	15.72	49.30	201.16
2	$w = 525.23 - 161.41DBH - 100.41ht - 20.30 \log DBH.ht$	7745.75	0.54	0.55	5.58×10^{-16}	258.19	16.06	50.38	204.84
3	$w = -34.46 + \frac{1.65DBH}{0.02DBH.ht}$	8836.56	0.47	0.49	5.57×10^{-16}	294.55	17.16	53.81	218.31
4	$w = -6.04 + 1.72ht^2 - 1.52DBH$	8868.51	0.47	0.49	-2.04×10^{-15}	295.61	17.19	53.91	218.61
5	$w = -6.04 + \frac{-1.52ht}{1.72DBH}$	8889.78	0.47	0.49	-1.85×10^{-15}	296.32	17.21	53.97	214.07

Where: SQE = Error sum of squares, r^2 = Determination coefficient, r^2_{ajus} = Adjusted determination coefficient, RMSE = Root mean squared error, x^2 = chi-square, Syx = Estimated standard error, and Syx% = Standard error of the estimated percentage.

Estimated biomass

Figure 5 demonstrates the application of the adjusted model to all trees measured in the plots concerning diameter and height. There is a higher concentration of trees in the classes up to 20 cm in diameter with biomass up to 200 kg; However, from the class with 20 cm, there is a smaller number of individuals with biomass above 200 kg per tree (Figure 5A). Regarding heights, there is a concentration of trees above 5 m and smaller than 15 m.

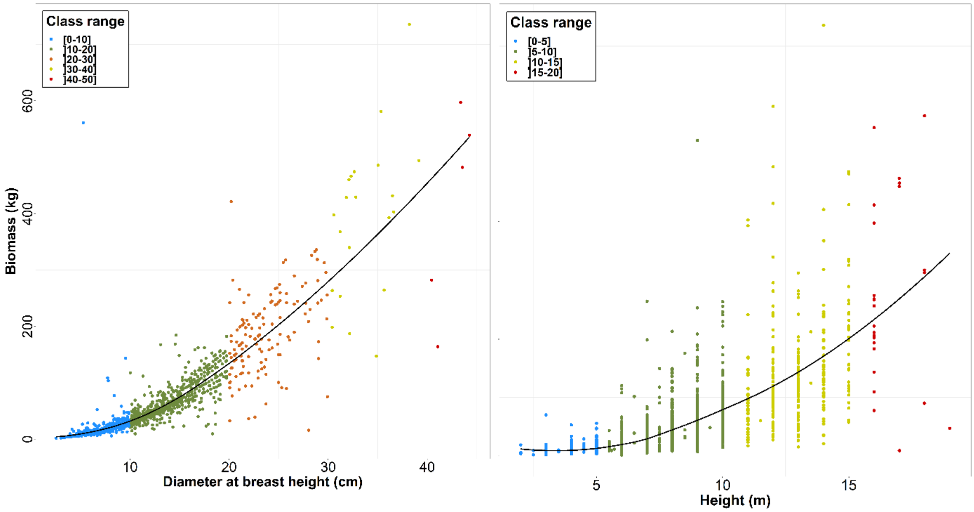

Figure 5. Biomass distribution concerning diameter and height class range.

Biomass stock in the plots

The prediction of biomass showed that, except for Plots 6, 7, 18, and 19, it was affected by an increasing trend in biomass stock between the years 2016 to 2020; However, in four plots (1, 2, 17, and 18) it was not possible to obtain data for all years of the inventory. The following growth percentages were observed between the years under study: Plot 3 (93.42 %), Plot 4 (30.39 %), Plot 5 (89.69 %), Plot 6 (25.96 %), Plot 7 (115.23 %), Plot 8 (73.39 %), Plot 9 (66.09 %), Plot 10 (94.32 %), Plot 11 (175.54 %), Plot 12 (123.87 %), Plot 13 (65.59 %), Plot 14 (141.46 %), Plot 15 (137.28 %), Plot 16 (113.84 %), Plot 18 (117.55 %), and Plot 19 (90.74 %) (Figure 6).

In 2016, the largest stock of biomass among the inventoried plots was in Plot 18 (55.42 $t.ha^{-1}$) and the smallest in Plot 14 (15.86 $t.ha^{-1}$), while in 2020, Plot 11 presented the largest stock (135.23 $t.ha^{-1}$), and the lowest value was 30.91 $t.ha^{-1}$ (Plot 6). It is observed that Plots 6, 15, 18, and 19 showed a reduction in their stock between 2019 and 2020 (-4.99, -2.25, -6.25, and -18.31 $t.ha^{-1}$, respectively).

Regarding the abundance of individuals per hectare, it was possible to observe that Plots 3, 12, 13, 16, and 19 were not affected by a reduction in individuals in 2019, while in the other Plots there was a decrease in the number of trees. The average number of trees in the plots was: 2016 (1,198 $trees.ha^{-1}$), 2018

(1,466 trees.ha^{-1}), 2019 (1,118 trees/ha), 2020 (1,468 trees.ha^{-1}). It is observed that Plot 8 has the greatest abundance (2,200 trees.ha^{-1}), while Plot 16 had only 738 trees.ha^{-1}.

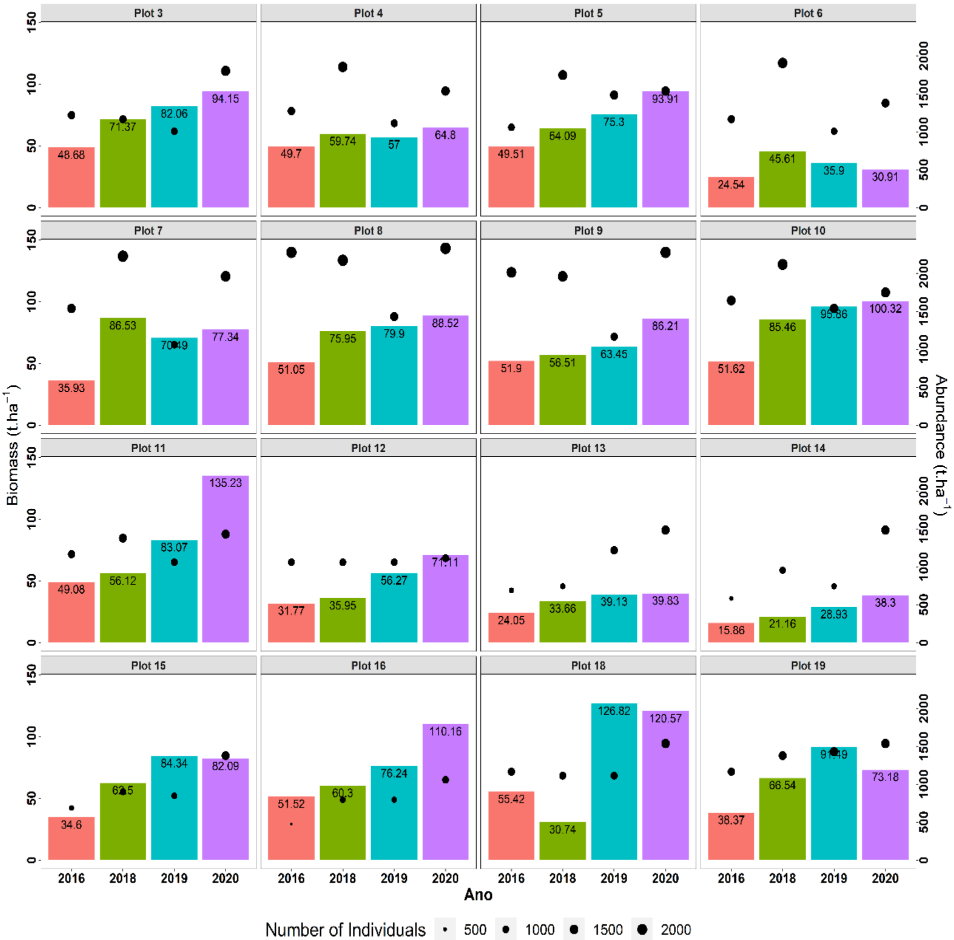

Figure 6. Biomass stock from plots inventoried in the years 2016 to 2020.

Figure 7 demonstrates the distribution of biomass stock in the plots concerning the years that the inventories took place. In 2016, the plots had an average of 41.47 t.ha^{-1} and a deviation of 12.29 t.ha^{-1}. 2018 presented a total of 57.02 t.ha^{-1} and 19.20 t.ha^{-1} deviation. 2019 presented an average 71.64 t.ha^{-1} and 24.77 t.ha^{-1} deviation. However, 2020 recorded an average of 81.66 t.ha^{-1} and 28.99 t.ha^{-1} deviation. In this sense, a total increase of 37.50% between 2016 and 2018 was observed, 25.64% from 2018 to 2019 and 13.99% from 2019 to 2020, therefore a total increase from 2016 to 2020 of 96.92% was observed.

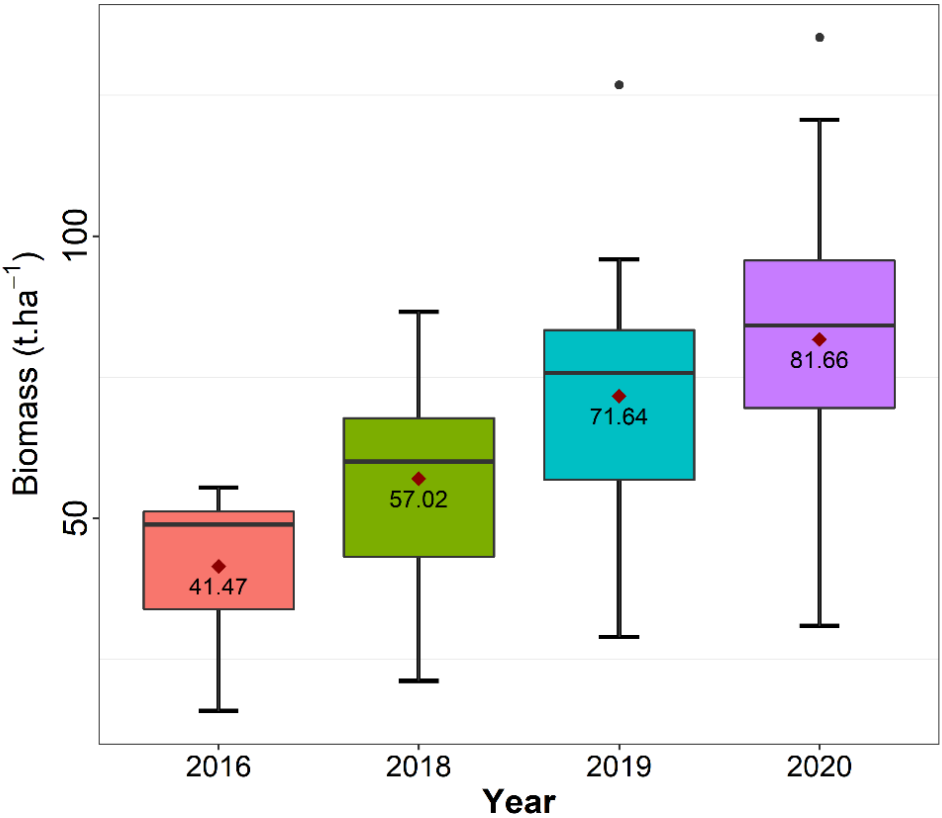

Figure 7. Total biomass stock of plots inventoried in the years 2016 to 2020.

DISCUSSION

The allocation and determination of biomass will play an important role based on previously degraded forests that have undergone restoration/recovery processes. In this context, the adjusted models predicting biomass from DBH and ht demonstrate that there is a low correlation between the dependent and independent variables. The adjusted correlation coefficients ranged from 0.49 to 0.57, which can be explained by the diversity of species sampled, increasing the variability of measured biomass, diameters, and heights among the sampled trees.

By analyzing Model 1, chosen among the five best ones, it was possible to observe that biomass prediction with this model was satisfactory because it presented values consistent with the area and with the values obtained in the field. However, Romero *et al.* (2020) observed superior statistical metrics when estimating the biomass of trees harvested in southWestern Amazonia and reported that the quality of the adjustments was characterized by the expressive quantity of samples collected (223 individuals). Karyati *et al.* (2019), observing secondary forests, showed similar results to this study and concluded that the heterogeneity of secondary forests significantly reduces the relationships between diameters, heights, and biomass.

In this sense, several factors influence the development of tree species in secondary forests; thus, characteristics such as canopy architecture, local productive capacity, and competition for light may be related to the low statistical values observed for the estimates made in this study. In addition to these factors, in native forests, forests undergoing restoration, and in mixed plantations, greater heterogeneity in tree development is to be expected, caused by the existence of interspecific competition, which contributes to obtaining standard error values for the highest estimate (Sanquetta et al. 2017; Lima et al. 2021).

The reduction observed in biomass stock in plots 6, 7, 18, and 19 is explained by the mortality of trees and illegal logging, which were responsible for the reduction of biomass in those plots, reducing trees density and consequently the total stock values. In particular, plots 18 and 19, which are on the transition with soybean crops, were affected by partial removal of forest vegetation for soybean implantation.

The results obtained in this study were consistent with those observed in Yang *et al.* (2020), where the authors estimated the aboveground biomass using remote sensing techniques for areas undergoing restoration with ages ranging from 1 to 8 years and biomass ranging from 20 to 70 $Mg.ha^{-1}$. Cassol *et al.* (2019) observed that areas in the Amazon undergoing a 10-year restoration process have less than 100 Mg/ha, with data obtained through Alos/Palsar-2. The difficulty in obtaining biomass in the field is a determining factor for this variable acquisition through remote sensors.

Studies show that the greater density of individuals in an area, the greater production of biomass per unit area; that is, the spacings between the denser individuals provide higher amounts of biomass than the smaller ones (Pereira 2013; Eloy et al. 2016; Favero et al. 2020; Castanho et al. 2020; Shen et al. 2020; Næsset et al. 2020). It was possible to observe in this study that Plots with lower abundances per hectare may also have biomass stocks equivalent to Plots with greater abundance as the size (height and DBH) of individuals directly influenced the mean value of biomass. In 2020, Plots 6 and 16 had an equal number of trees per hectare (1,300 $trees.ha^{-1}$) but biomass was respectively 30.91 and 82.09 $t.ha^{-1}$. It links to the average diameters and heights of trees in the plots, which was higher for Plot 15 with an average DBH of 15.6 cm and an average height of 9.4 m, while in Plot 6 averages were 12.8 cm and 7.4 m.

The information presented regarding secondary forests is essential for the monitoring and management of these areas because it subsidizes policies and proposals for environmental management in the Amazon. These forests are a possible solution for carbon absorption and climate change control, besides conserving biodiversity. Therefore, the reduction of deforestation, agriculture, and cattle raising allied to the maintenance of forest areas are vital to mitigate the impacts caused by climate change.

CONCLUSIONS

The adjusted models using diameter at breast height (DBH) and total height (ht) as independent variables present satisfactory statistics and can be used to estimate tree biomass with precision.

The results demonstrate that few trees with large dimensions are responsible for biomass stock in areas under restoration, while the abundance of individuals per hectare influences biomass stock. However, the main factor in these stocks were heights and diameters; in this sense, the larger the trees, the greater biomass stocks.

Through the biomass estimates, it was possible to observe that secondary forests are a potentially significant biomass sink due to the rapid accumulation rates of this component. Therefore, biomass stocks were increased over the years of study, demonstrating the capacity for biomass growth in forests undergoing restoration. However, frequent disturbances interrupt the recovery process and consequently the establishment of mature forests.

The value of secondary forests as a biomass stock needs to be determined within a context of dynamic land use, assessing stocks in primary forests concerning secondary forests and areas with other land uses.

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REFERENCES

Barlow J, Gardner TA, Araujo IS, et al (2007) Quantifying the biodiversity value of tropical primary, secondary, and plantation forests. Proc Natl Acad Sci U S A 104:18555–18560. https://doi.org/10.1073/pnas.0703333104

Barros TC, Elias F, Romano LL, Ferreira J (2020) Natural recovery of plant species diversity in secondary forests in Western Amazonia: contributions to passive forest restoration. Rev Bras Bot 43:165–175. https://doi.org/10.1007/s40415-020-00585-9

Carvalho R, Adami M, Amaral S, et al (2019): Changes in secondary vegetation dynamics in a context of decreasing deforestation rates in Pará Brazilian Amazon. Appl Geogr 106:40–49. https://doi.org/10.1016/j.apgeog.2019.03.001

Cassol HLG, Carreiras JM de B, Moraes EC, et al (2019): Remote sensing Retrieving Secondary Forest Aboveground Biomass from Polarimetric ALOS-2 PALSAR-2 Data in the Brazilian Amazon. 11:59. https://doi.org/10.3390/rs11010059

Castanho ADA, Coe MT, Brando P, et al (2020): Potential shifts in the aboveground biomass and physiognomy of a seasonally dry tropical forest in a changing climate. Environ Res Lett 15:034053. https://doi.org/10.1088/1748-9326/ab7394

Chave J, Andalo C, Brown S, et al (2005): Tree allometry and improved estimation of carbon stocks and balance in tropical forests. Oecologia 145:87–99. https://doi.org/10.1007/s00442-005-0100-x

Chazdon RL, Broadbent EN, Rozendaal DMA, et al (2016): Carbon sequestration potential of second-growth forest regeneration in the Latin American tropics. Sci Adv 2:. https://doi.org/10.1126/sciadv.1501639

Corte APD, Souza DV, Rex FE, et al (2020): Forest inventory with high-density UAV-Lidar: Machine learning approaches for predicting individual tree attributes. Comput Electron Agric 179:105815. https://doi.org/10.1016/j.compag.2020.105815

Eloy E, Silva DA da, Schmidt D, et al (2016): EFFECT OF PLANTING AGE AND SPACING ON ENERGY PROPERTIES OF Eucalyptus grandis W. Hill EX Maiden. Rev Árvore 40:749–758. https://doi.org/10.1590/0100-67622016000400019

Favero A, Daigneault A, Sohngen B (2020): Forests: Carbon sequestration, biomass energy, or both? Sci Adv 6:eaay6792. https://doi.org/10.1126/sciadv.aay6792

Feng Y, Lu D, Chen Q, et al (2017): Examining effective use of data sources and modeling algorithms for improving biomass estimation in a moist tropical forest of the Brazilian Amazon. Int J Digit Earth 10:996–1016. https://doi.org/10.1080/17538947.2017.1301581

Karyati, Ipor IB, Jusoh I, Wasli ME (2019): Allometric equations to estimate the above-ground biomass of trees in the tropical secondary forests of different ages. Biodiversitas 20:2427–2436. https://doi.org/10.13057/biodiv/d200901

Kenzo, T., Himmapan W, Yoneda R, et al (2020): General estimation models for above- and below-ground biomass of teak (Tectona grandis) plantations in Thailand. For Ecol Manage 457:117701. https://doi.org/10.1016/j.foreco.2019.117701

Latifah, S., Purwoko A, Sri Hartini K, Amalia Fachrudin K (2021): Allometric models to estimate the aboveground biomass of forest: A literature review. IOP Conf Ser Mater Sci Eng 1122:012047. https://doi.org/10.1088/1757-899X/1122/1/012047

Lima RC, Sardinha MA, Souza J dos S, et al (2021): Composition and structure of a stretch of tropical forest in the Western Amazon. Ciência Rural 51:2021. https://doi.org/10.1590/0103-8478cr20200312

Matos FAR, Magnago LFS, Aquila Chan Miranda C, et al (2019): Secondary forest fragments offer important carbon and biodiversity cobenefits. Glob Chang Biol 26:509–522. https://doi.org/10.1111/gcb.14824

Mitchell AL, Rosenqvist A, Mora B (2017): Current remote sensing approaches to monitoring forest degradation in support of countries measurement, reporting and verification (MRV) systems for REDD+. Carbon Balance Manag. 12

Mohd Zaki NA, Abd Latif Z (2017): Carbon sinks and tropical forest biomass estimation: a review on role of remote sensing in aboveground-biomass modelling. Geocarto Int. 32:701–716

Næsset E, McRoberts RE, Pekkarinen A, et al (2020): Use of local and global maps of forest canopy height and aboveground biomass to enhance local estimates of biomass in miombo woodlands in Tanzania. Int J Appl Earth Obs Geoinf 93:102138. https://doi.org/10.1016/j.jag.2020.102138

Ngomanda A, Engone Obiang NL, Lebamba J, et al (2014): Site-specific versus pantropical allometric equations: Which option to estimate the biomass of a moist central African forest? For Ecol Manage 312:1–9. https://doi.org/10.1016/j.foreco.2013.10.029

Nunes S mia, Oliveira L, Siqueira J o., et al (2020): Unmasking secondary vegetation dynamics in the Brazilian Amazon. Environ Res Lett 15:034057. https://doi.org/10.1088/1748-9326/ab76db

Pereira MG (2013): A seção de método de um artigo científico. Epidemiol e Serviços Saúde 22:183–184. https://doi.org/10.5123/S1679-49742013000100020
Poorter L, Bongers F, Aide TM, et al (2016): Biomass resilience of Neotropical secondary forests. Nature 530:211–214. https://doi.org/10.1038/nature16512
Romero FMB, Jacovine LAG, Ribeiro SC, et al (2020): Allometric Equations for Volume, Biomass, and Carbon in Commercial Stems Harvested in a Managed Forest in the Southwestern Amazon: A Case Study. Forests 11:874. https://doi.org/10.3390/f11080874
Saha C, Mahmood H, Nayan SNS, et al (2021): Allometric biomass models for the most abundant fruit tree species of Bangladesh: A Non-destructive approach. Environ Challenges 3:100047. https://doi.org/10.1016/j.envc.2021.100047
Sanquetta CR, Behling A, Corte APD, et al (2014a): Eficiência de conversão da radiação fotossintética interceptada em Fitomassa de mudas de Eucalyptus dunii Maiden em função da densidade de plantas e do ambiente de cultivo. Sci For Sci 42:573–580
Sanquetta CR, Behling A, Dalla Corte AP, et al (2014b): A model based on environmental factors for diameter distribution in black wattle in Brazil. PLoS One 9:. https://doi.org/10.1371/journal.pone.0100093
Sanquetta CR, Sanquetta MNI, Bastos A, et al (2017): ESTIMATIVA DA ALTURA E DO VOLUME EM POVOAMENTOS JOVENS DE RESTAURAÇÃO FLORESTAL EM RONDÔNIA. BIOFIX Sci J 2:23. https://doi.org/10.5380/biofix.v2i2.54124
Shen G, Wang Z, Liu C, Han Y (2020): Mapping aboveground biomass and carbon in Shanghai's urban forest using Landsat ETM+ and inventory data. Urban For Urban Green 51:126655. https://doi.org/10.1016/j.ufug.2020.126655
Teixeira-Santos J, da Cunha Ribeiro AC, Wiig Ø, et al (2020): Environmental factors influencing the abundance of four species of threatened mammals in degraded habitats in the Western Brazilian Amazon. PLoS One 15:1–16. https://doi.org/10.1371/journal.pone.0229459
Tejada G, Görgens EB, Ovando A, Ometto JP (2020): Mapping data gaps to estimate biomass across Brazilian Amazon forests. For Ecosyst 7:25. https://doi.org/10.1186/s40663-020-00228-1
Tripathi P, Patel NR, Kushwaha SPS (2018): Estimating net primary productivity in tropical forest plantations in India using satellite-driven ecosystem model. Geocarto Int 33:988–999. https://doi.org/10.1080/10106049.2017.1323963
Van Breugel M, Ransijn J, Craven D, et al (2011): Estimating carbon stock in secondary forests: Decisions and uncertainties associated with allometric biomass models. For Ecol Manage 262:1648–1657. https://doi.org/10.1016/j.foreco.2011.07.018
Vinh T Van, Marchand C, Linh TVK, et al (2019): Allometric models to estimate above-ground biomass and carbon stocks in Rhizophora apiculata tropical managed mangrove forests (Southern Viet Nam). For Ecol Manage 434:131–141. https://doi.org/10.1016/j.foreco.2018.12.017
Virgulino-Júnior PCC, Carneiro DN, Nascimento WR, et al (2020): Biomass and carbon estimation for scrub mangrove forests and examination of their allometric associated uncertainties. PLoS One 15:e0230008. https://doi.org/10.1371/journal.pone.0230008

Wang R, Gamon JA, Emmerton CA, et al (2020a): Detecting intra- and inter-annual variability in gross primary productivity of a North American grassland using MODIS MAIAC data. Agric For Meteorol 281:107859. https://doi.org/10.1016/j.agrformet.2019.107859

Wang Y, Ziv G, Adami M, et al (2020b) Upturn in secondary forest clearing buffers primary forest loss in the Brazilian Amazon. Nat Sustain 3:290–295. https://doi.org/10.1038/s41893-019-0470-4

Yang Y, Saatchi S, Xu L, et al (2020) Interannual Variability of Carbon Uptake of Secondary Forests in the Brazilian Amazon (2004-2014). Global Biogeochem Cycles 34:1–14. https://doi.org/10.1029/2019GB006396

Zhou X, Yang M, Liu Z, et al (2021) Dynamic allometric scaling of tree biomass and size. Nat Plants 7:42–49. https://doi.org/10.1038/s41477-020-00815-8

Oltenacu, C.V.., Oltenacu, N. (2022): The use of Zingiber officinale (ginger) extract for the control of Alternaria alternata fungal pathogen. Agriculture and Forestry, 68 (1): 25-40. doi:10.17707/AgricultForest.68.1.02

DOI: 10.17707/AgricultForest.68.1.02

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THE USE OF *ZINGIBER OFFICINALE* (GINGER) EXTRACT FOR THE CONTROL OF *ALTERNARIA ALTERNATA* FUNGAL PATHOGEN

SUMMARY

This study presents the treatment of pepper seeds with ginger extract for their germination and emergence, as well as the reduction of pesticides using alternative non-polluting methods to control pathogens.

The observations followed both the germination and emergence of the seeds, as well as the development of the pathogen *Alternaria alternata* and the appearance of necrosis and symptoms of alternariosis. Numerous data from the literature offer recommendations on the biological and chemical control of *A. alternata*, however more frequently there is the problem of maintaining their effectiveness and the detection of new products that prevent and combat the pathogen.

This research states that the ginger stimulates seed germination and has both preventive and curative action against the pathogen *A. alternata*.

Keywords: extract, ginger, pathogen, pepper

INTRODUCTION

Ginger (*Zingiber officinale*) is a herbaceous plant found in the tropics, which has an aromatic rhizome, rich in essential oils. It is highly valued as a spice and is used in all types of dishes, from sauces, soups and main courses, to desserts and drinks. Moreover, it is used since antiquity in medicine, to relieve nausea, relieve cramps and improve blood circulation.The rhizome of *Z. officinale* contains pungent phenolic substances, with a variety of biological activities (Wang *et al*., 2014).

It was shown that the consumption of ginger could influence the weight loss, by decreasing the body mass index and serum insulin (Ebrahimzadeh Attari *et al*., 2016.)

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Researchers have found that gingerol, the main bioactive compound, would inhibit the growth of several types of bacteria (Park et al., 2008; Karuppiah and Rajaram, 2012). In addition to gingerol, ginger contains large amounts of vitamin C and B6, along with magnesium, potassium, copper, manganese. Gingerols and their corresponding dehydration products shogaols were considered as the active compounds of ginger, the rhizome of the plant *Zingiber officinale*, for its antioxidant, anti-inflammatory, anti-diabetic and antitumor activities (Feng *et al*., 2011; Poltronieli *et al*., 2014; Khandouzi *et al.*, 2015). It is also used in the perfume and cosmetics industry, for hair toning and revitalization, ubiquitous in the revitalizing formulas of face creams.

There has been a growing global concern about the use of chemicals in the cultivation of vegetables, flowers, fruit trees and vines, due to their effects on human health and the environment. Fungicides from natural extracts have proven to be extremely useful in controlling plant pathogens, being a future alternative to synthetic fungicides.

Recent studies have shown that ginger oil has a very strong antimicrobial and therapeutic effect (Imamović *et al*., 2021).

This study presents the treatment of pepper seeds with ginger extract for their germination and emergence, as well as the reduction of pesticides using alternative non-polluting methods to control pathogens.

The observations followed both the germination and emergence of the seeds, as well as the development of the pathogen *Alternaria alternata* and the appearance of necrosis and symptoms of alternariosis.

Numerous data from the literature offer recommendations on the biological (Feng and Zheng, 2007; Tozlu *et al*., 2018) and chemical (Kapsa, 2009) control of the pathogen *Alternaria alternata*, however more frequently there is the problem of maintaining their effectiveness and the detection of new products that prevent and combat the pathogen.

This paper aimed to test the effect of ginger extract in different concentrations, on seed germination and seedling emergence and also to estimate by seed tests the effectiveness of *Zingiber officinale* in different concentrations, against the pathogen *Alternaria alternata*.

MATERIAL AND METHODS

The research was carried out in the chemistry laboratory within the Research and Development Station for Fruit Tree Growing Baneasa, Bucharest.

Plant material and pathogenic fungus. In order to test and estimate the effectiveness of ginger - *Zingiber officinale* in different concentrations, both on the germination and emergence of plants and against the pathogen *Alternaria alternata*, Romanian variety Barbara (Agrosel) of pepper seeds was used.

The ginger extract was obtained by pressing the fresh rhizome of *Z. officinale* followed by obtaining different concentrations (1%, 5% and 10%).

Alternaria alternata isolated from diseased fruits and seeds was the pathogenic species used to estimate the efficacy of ginger in different concentrations by seed tests.

The substance with fungicidal action tested was in the form of fresh ginger extract.

The blotch test method, recommended by International Seed Testing Association was used to test the three experiments: the pepper seeds were placed between the folds of blotting paper moistened with sterile distilled water, followed by their incubation at temperatures between 22-25°C for 3-6-9 days (Malone and Muskett, 1964; Hulea, 1969; Rădulescu and Negru, 1966).

Method used to study the influence of ginger treatment in different concentrations on pepper germination and emergence. A number of 60 seeds for each variant (1%, 5%, 10%) were washed with sterile distilled water, dried in an oven, and then moistened by immersion for 15 minutes with ginger (Figure 1). The control group was composed of 60 seeds, washed with sterile distilled water and then dried (Baicu, 1968; Constantinescu, 1974). Observations were performed in dynamics (three, six and nine days) and followed seed germination and the emergence of pepper plantlets.

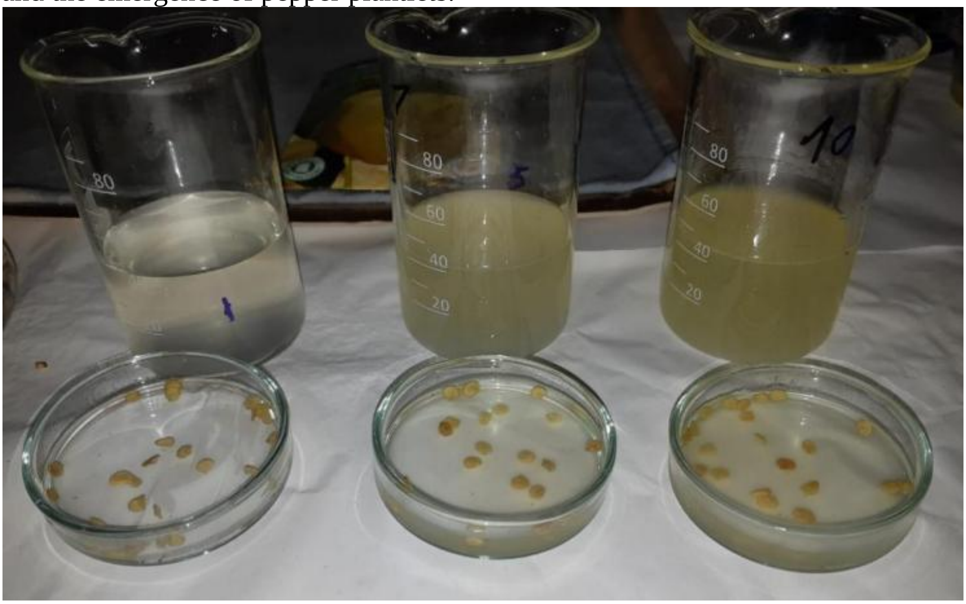

Figure 1. The appearance of ginger-treated seeds in different concentrations

Method used to estimate the effectiveness of ginger treatments in different concentrations in order to prevent and control the pathogenic species *Alternaria alternata in vitro* by seed tests. The tested pathogen was isolated directly from the plant material. The plant material was passed through distilled water twice. Inoculum harvesting was performed by detaching small portions of mycelium from the affected areas with pathogens that had the characteristic aspect of mycelium. The detached mycelium was then placed on PDA culture medium (Potato-glucose-agar, Difco, prepared according to the recipe, sterilized by autoclave: 1.2 atmospheres, 20 minutes). The harvesting operation was carried out in the sterile chamber-hood with laminar flow; with the scalpel, flamed and cooled, the inoculum was passed with the help of a transplanting needle on an isolating medium, sinking slightly.

Incubation was performed at 24°C for 10 days; the developed colonies were purified by transferring to another vessel with sterile culture medium. Isolates were identified based on spore morphology, then transplanted and stored in pure cultures at 24°C (Manole and Ciocoiu, 2011).

In order to estimate the effectiveness of treatments with ginger in different concentrations in the control of *Alternaria alternata*, two variants were used:

Pre-treatment. Overall, 60 seeds (for each variant - 1%, 5%, 10%) were disinfected with 70% ethyl alcohol, washed with sterile distilled water, dried in an oven, then moistened by immersion for 15 minutes with a mixture of ginger and artificially contaminated by immersion in a calibrated spore suspension (5×10^6 spores / ml) for one hour. The contaminated seeds were incubated at 25°C.

Treatment. A number of 60 seeds (for each variant - 1%, 5%, 10%) were disinfected with 70% ethyl alcohol, washed with sterile distilled water, dried in an oven, then artificially contaminated, by bathing in a calibrated suspension of spores (5×10^6 spores / ml) for one hour. The seeds were then moistened, by immersing them, for 15 minutes, with ginger in different concentrations. The contaminated seeds were incubated at 25°C. The observations followed both the development of seedlings and the appearance of necrosis and alternariosis symptoms (Iacomi et al., 2004).

RESULTS AND DISCUSSION

Influence of ginger treatment (concentration 1%, 5%, 10%) on pepper germination and germination. In the control group (Figure 2) the germination was registered as 65% (on day 9), the seeds developed root/hypocotyl/cotyledons, but the seedlings were covered with *Rhizopus* sp. and *Aspergillus* sp.

The ginger variant - *Zingiber officinale* - concentration 1% allowed a germination, on day 9, of 70% (at 61% the root and the hypocotyl have developed, the rest presenting the root, hypocotyl and cotyledons - Figures 3, 4).

The ginger - *Zingiber officinale* - in a concentration of 1% did not influence the germination of seeds and did not allow the development of saprophytic or pathogenic fungi.

Figure 2. The untreated control group

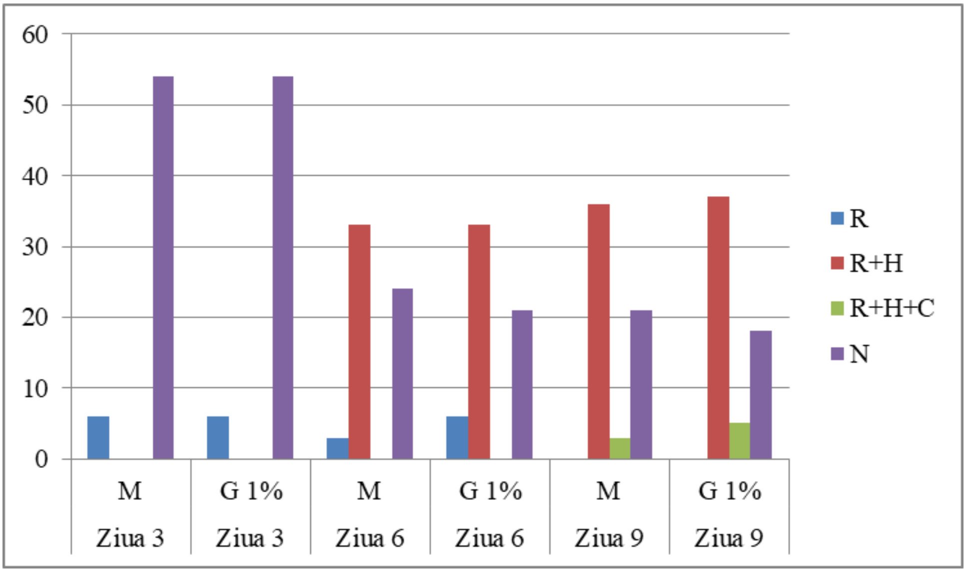

Figure 3. The influence of ginger treatment - Zingiber officinale - 1% concentration, on pepper germination and emergence M- control group, G 1% - ginger extract 1%, Ziua - Day, N=ungerminated, R = radicule, H = hypocotyl, C = cotyledons

Figure 4. The influence of ginger treatment - *Zingiber officinale* - 1% concentration, on pepper germination and emergence

The influence of ginger treatment - *Zingiber officinale* - 5% concentration, on pepper germination and emergence. In the control group, the seeds germinated in 65% on day 9 (Figure 5), they developed root/hypocotyl/cotyledons but the seedlings were covered with *Rhizopus* sp., *Aspergillus* sp.

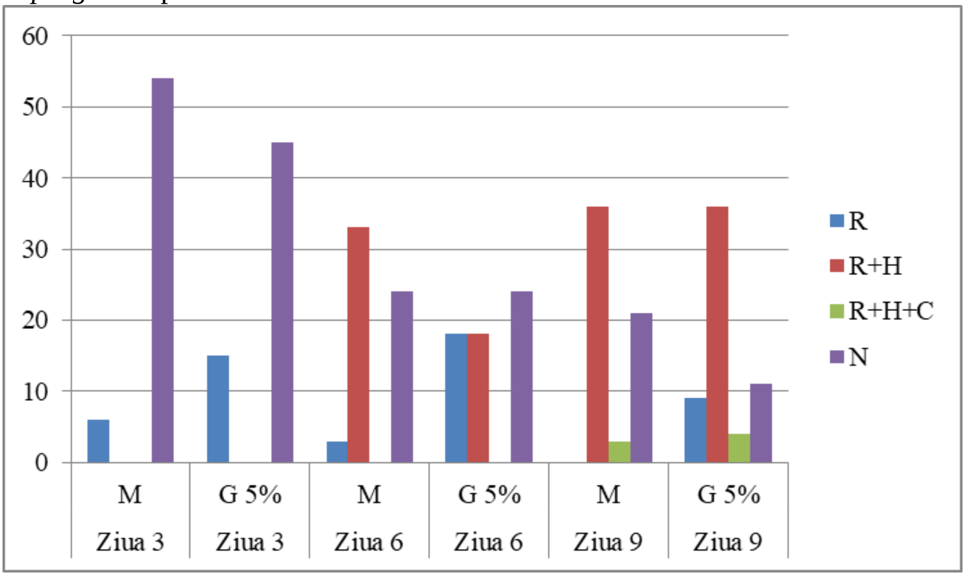

Figure 5. The influence of ginger treatment - Zingiber officinale - 5% concentration, on pepper germination and emergence M- control group, G 1% - ginger extract 1%, Ziua - Day, N=ungerminated, R = radicule, H = hypocotyl, C = cotyledons

The ginger - *Zingiber officinale* - in a concentration of 5% allowed a germination of 81.66%, on day 9 (Figure 6). This variant has favorably influenced the germination and development of the seedlings, compared to the control group, and did not allow the development of saprophytic or pathogenic fungi.

Figure 6. The influence of ginger treatment - Zingiber officinale - 5% concentration, on pepper germination and emergence

The influence of ginger treatment - *Zingiber officinale* - 10% concentration, on pepper germination and emergence. In the control group the rated of germination was 65% in day 9 (Figure 7), they developed root/hypocotyl/cotyledons and the plantlets were covered with *Rhizopus* sp. and *Aspergillus* sp.

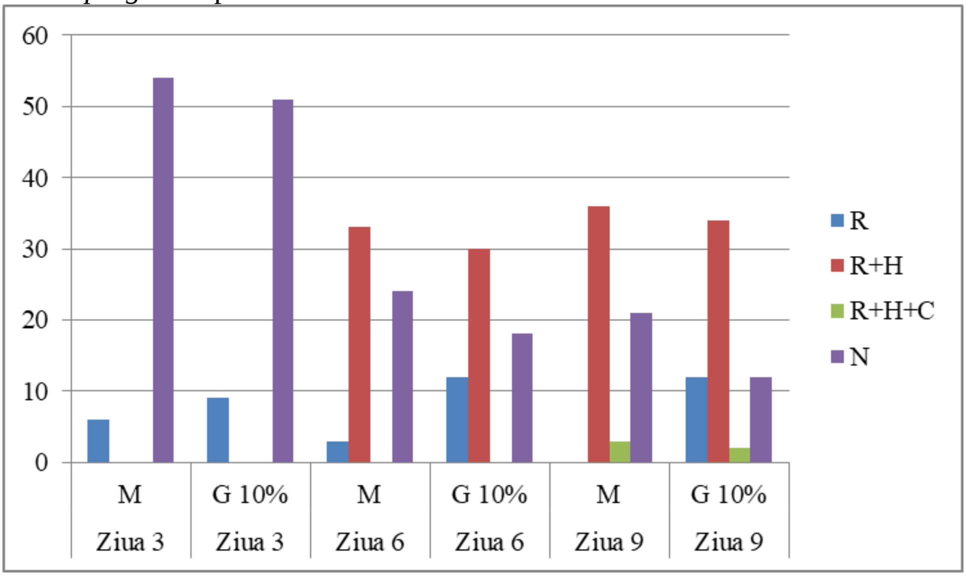

Figure 7. The influence of ginger treatment - Zingiber officinale - 10% concentration, on pepper germination and emergence M- control group, G 1% - ginger extract 1%, Ziua - Day, N=ungerminated, R = radicule, H = hypocotyl, C = cotyledons

The ginger variant - *Zingiber officinale* - 10% concentration allowed a germination of 80%, on day 9 (Figure 8). This variant did not allow the development of saprophytic or pathogenic fungi either.

Figure 8. The influence of ginger treatment - Zingiber officinale - 10% concentration, on pepper germination and emergence

Compared to the control group, where the germination was 65% and the seedlings were well developed (root, hypocotyl, cotyledons), the variants 1%, 5%, 10% of ginger - *Zingiber officinale*, positively influenced the germination, reaching up to 81.66% in variant 5%.

Efficiency of preventive treatment with ginger - *Zingiber officinale* - 1% concentration in combating the pathogen *Alternaria alternata* in peppers. The infected, untreated control group germinated in a percentage of 33.33%, on day 9 having necrotic roots, hypocotyl and cotyledons, the seeds (Figures 9, 10) being covered with a black mycelium (*A. alternata*).

Due to the efficiency of the action of ginger - *Zingiber officinale,* in the 1% concentration variant, the mycelium of the pathogenic fungus *Alternaria alternata* did not develop, and the seeds germinated in a proportion of 60%.

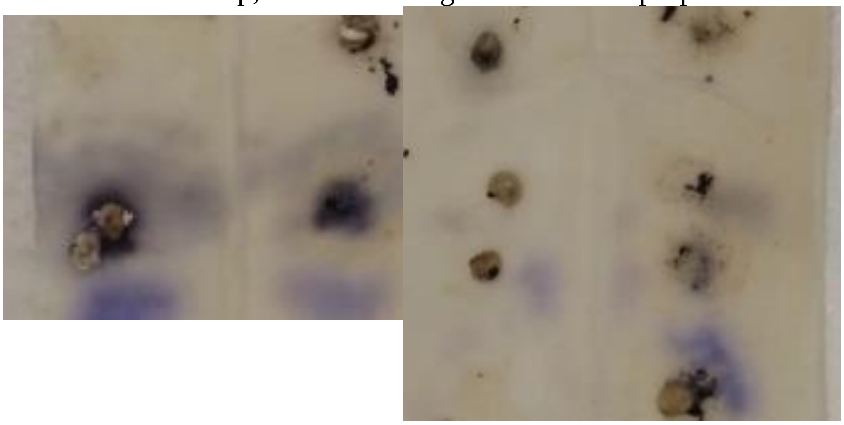

Figure. 9 The infected control group

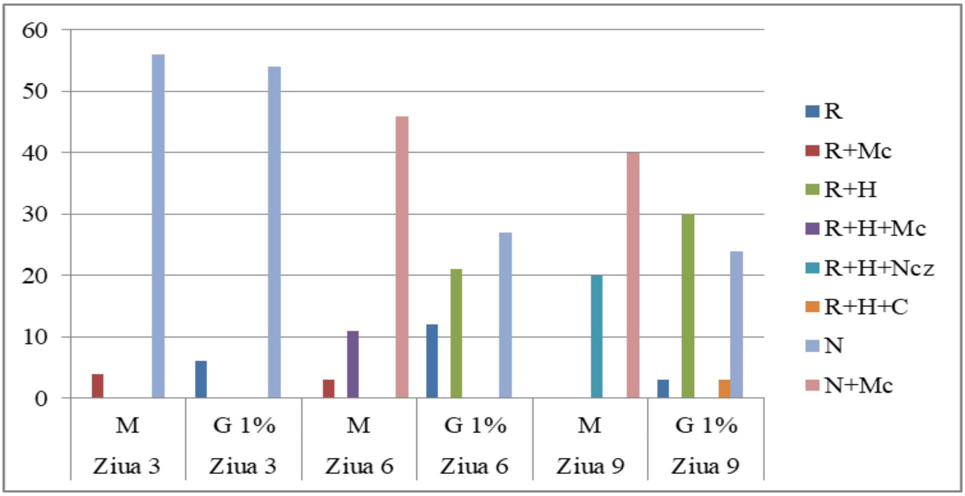

Figure 10. The efficiency of preventive treatment with ginger - Zingiber officinale - 1% concentration in the control of the pathogen Alternaria alternata in peppers (M – control group, G 1% - ginger extract 1% (Zingiber officinale), R = radicule, Mc = the presence of mycelium, H = hypocotyl, Ncz = necrosis, C = cotyledons, N =ungerminated seeds)

Efficiency of preventive treatment with ginger - *Zingiber officinale* - 5% concentration in combating the pathogen *Alternaria alternata* in peppers. The untreated, untreated control germinated in a percentage of 33.33%, on day 9 having necrotic roots, hypocotyl and cotyledons, the seeds (Figure 11) being covered with black mycelium (*Alternaria alternata*).

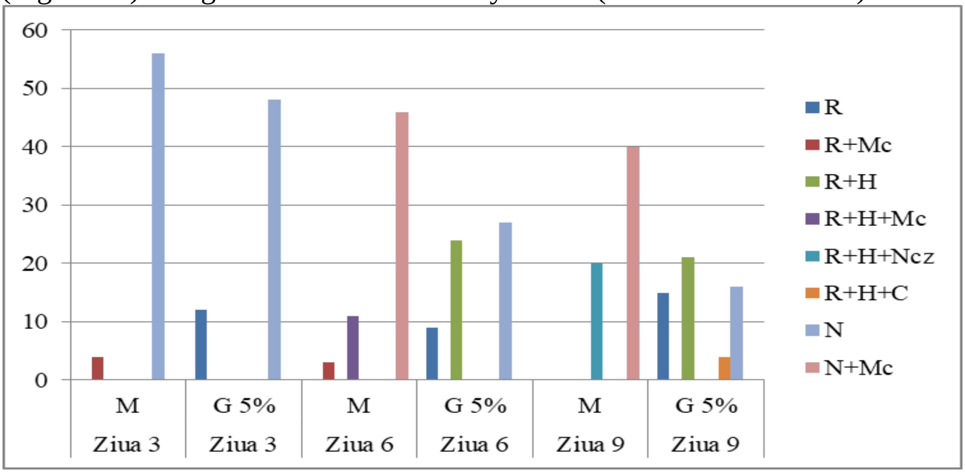

Figure 11. The efficiency of preventive treatment with ginger - Zingiber officinale - 5% concentration in the control of the pathogen Alternaria alternata in peppers (M – control group, G 5% - ginger extract 5% (Zingiber officinale), R = radicule, Mc = the presence of mycelium, H = hypocotyl, Ncz = necrosis, C = cotyledons, N =ungerminated seeds)

Due to the efficiency of the action of ginger - *Zingiber officinale*, in the 5% concentration variant, the mycelium of the pathogenic fungus *Alternaria alternata* did not develop, and the seeds had a germination proportion of 73.33%

Efficiency of preventive treatment with ginger - *Zingiber officinale* - 10% concentration in combating the pathogen *Alternaria alternata* in peppers. The infected, untreated control germinated in a percentage of 33.33%, on day 9 having necrotic roots, hypocotyl and cotyledons, the seeds (Figure 12) being covered with black mycelium (*Alternaria alternata*).

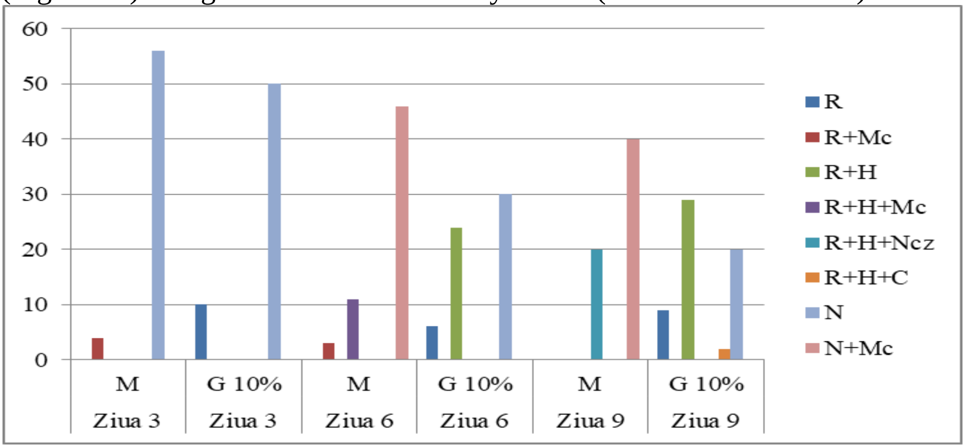

Figure 12. The efficiency of preventive treatment with ginger - Zingiber officinale - 10% concentration in the control of the pathogen Alternaria alternata in peppers (M – control group, G 5% - ginger extract 10% (Zingiber officinale), R = radicule, Mc = the presence of mycelium, H = hypocotyl, Ncz = necrosis, C = cotyledons, N =ungerminated seeds)

Due to the efficiency of the action of ginger, in the 10% concentration variant, the mycelium of the pathogenic fungus *Alternaria alternata* did not develop, and the seeds germinated in proportion of 66.66%.

Efficiency of curative treatment with ginger - *Zingiber officinale* - 1% concentration in combating the pathogen *Alternaria alternata* in peppers. The infected, untreated control germinated in a percentage of 33.33%, on day 9 having necrotic roots, hypocotyl and cotyledons, the seeds (Figure 13) being covered with black mycelium (*Alternaria alternata*).

The curative treatment with ginger - *Zingiber officinale*, in a concentration of 1%, in order to control the pathogen *Alternaria alternata* in peppers allowed a germination of 83.33%, and no development of the pathogenic fungi was registered (Figures 13, 14).

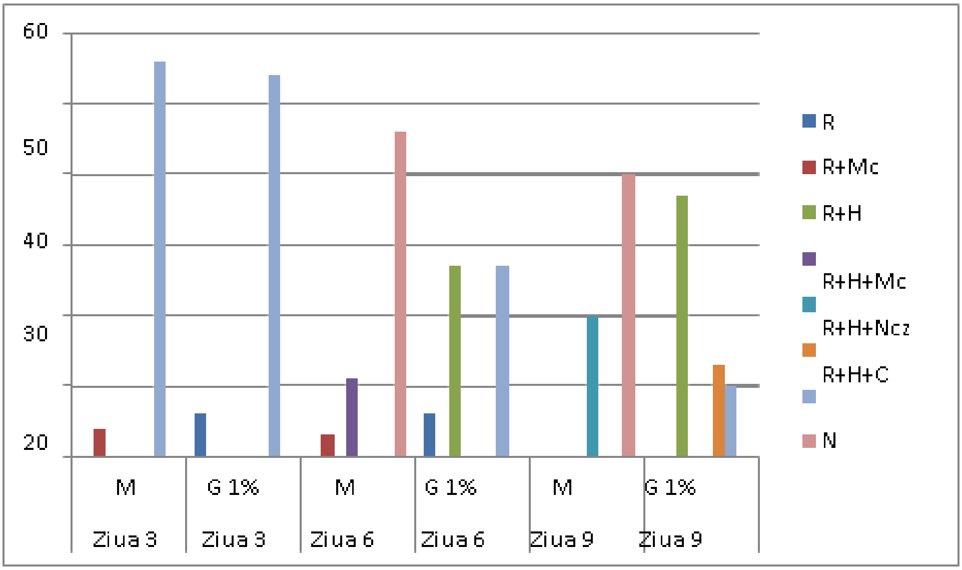

Figure 13. The efficiency of curative treatment with ginger - *Zingiber officinale* - 1% concentration in the control of the pathogen *Alternaria alternata* in peppers (M – control group, G 1% - ginger extract 1% (*Zingiber officinale*), R = radicule, Mc = the presence of mycelium, H = hypocotyl, Ncz = necrosis, C = cotyledons, N =ungerminated seeds)

Figure 14. The efficiency of curative treatment with ginger - *Zingiber officinale* - 1% concentration in the control of the pathogen *Alternaria alternata* in peppers (day 9)

The efficiency of curative treatment with ginger - *Zingiber officinale* - 5% concentration in the control of the pathogen *Alternaria alternata* in peppers (day 9) (Figures 15-16).

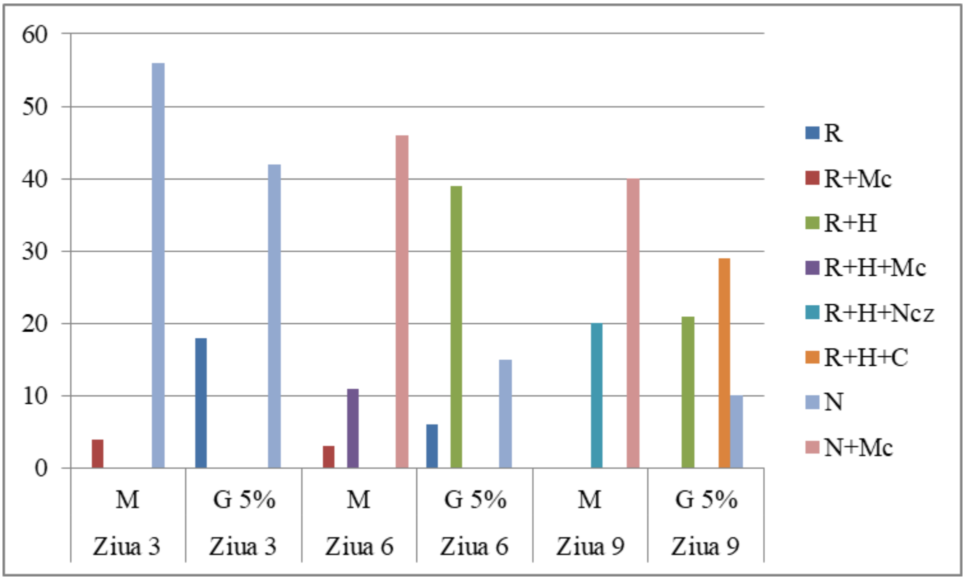

Figure 15. The efficiency of curative treatment with ginger - Zingiber officinale - 5% concentration in the control of the pathogen Alternaria alternata in peppers (M – control group, G 1% - ginger extract 5% (Zingiber officinale), R = radicule, Mc = the presence of mycelium, H = hypocotyl, Ncz = necrosis, C = cotyledons, N =ungerminated seeds)

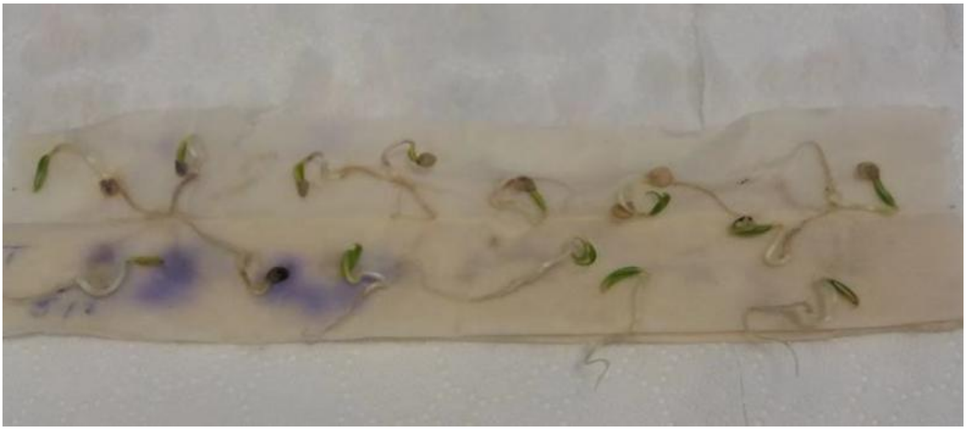

Figure 16. The efficiency of curative treatment with ginger - *Zingiber officinale* - 5% concentration in the control of the pathogen *Alternaria alternata* in peppers (day 9)

Efficiency of curative treatment with ginger - *Zingiber officinale* - 10% concentration in combating the pathogen *Alternaria alternata* in peppers. The curative treatment with ginger, in a concentration of 10%, in order to control the pathogen *Alternaria alternata* in peppers allowed a germination of 93.33%

(18.33% - root development, 61.66% - root and hypocotyl development, respectively 13.33% development of the root, hypocotyl and cotyledons) not allowing the pathogenic fungus to develop (Figures 17, 18).

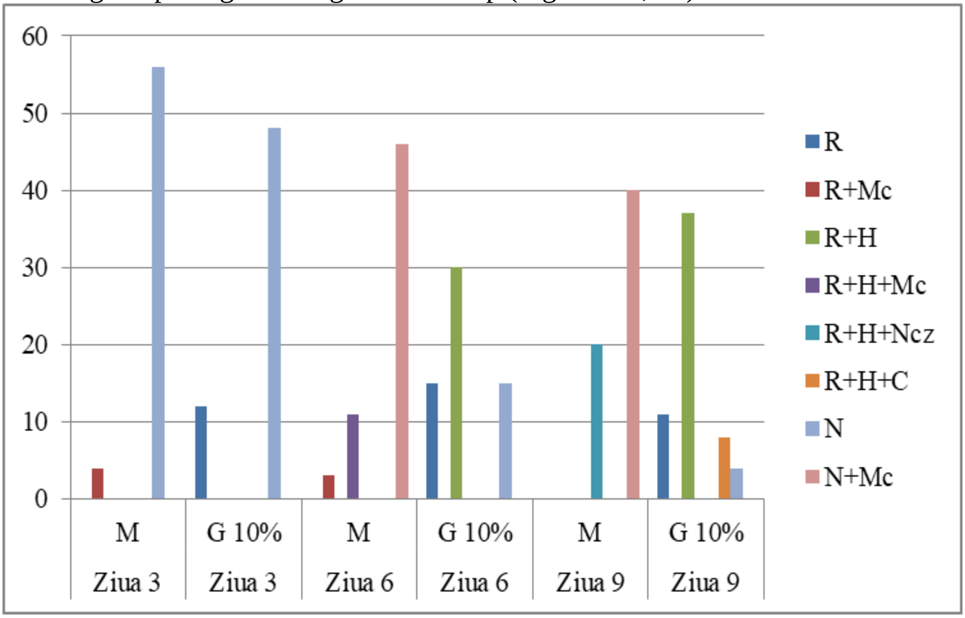

Figure 17. The efficiency of curative treatment with ginger - Zingiber officinale - 10 % concentration in the control of the pathogen Alternaria alternata in peppers (M – control group, G 1% - ginger extract 10 % (Zingiber officinale), R = radicule, Mc = the presence of mycelium, H = hypocotyl, Ncz = necrosis, C = cotyledons, N =ungerminated seeds)

Figure 18. The efficiency of curative treatment with ginger - *Zingiber officinale* - 10 % concentration in the control of the pathogen *Alternaria alternata* in peppers

Similar research (Sharma and Tiwari, 2013) highlighted the effectiveness of essential oil extracted from ginger (*Zingiber officinale* Roscoe.) in controlling *Alternaria alternata,* the minimum inhibitory concentration (MIC) of this oil showing results at 500 ppm for the fungus. Moreover, it was observed that essential oil extracted from ginger has a wide range of antifungal activities against *Aspergillus niger* van Tiegh., *Penicillium chrysogenum* Thom., *Alternaria alternata* (Fr.) Keissl. and *Fusarium roseum* Link., whereas MIC showed results at 1.0 mL/cm^{-3} of oil in respect to all fungi (Sharma *et al*., 2013).

The results of present work are consistent with other studies (Fawzi *et al.,* 2009; Osman *et al.,* 2016; Ahmad and Qureshi, 2017), which proved that ginger extracts have antibacterial activity against *A. alternata* fungus.

CONCLUSIONS

The present study has shown the favorable influence of ginger extract - *Zingiber officinale* on the germination of pepper seeds. All three variants of concentration (1%, 5% and 10%) had higher germination percentages - 66%, 70% and 80% respectively compared to the control group that germinated in a rate of 65%.

The preventive treatments with the three concentration variants (1%, 5%, 10%) inhibited the development of the pathogen *Alternaria alternata*.

The curative treatments with the three concentration variants (1%, 5%, 10%) also inhibited the development of the pathogen *A. alternata*. In this case, a pronounced stimulation of seed germination was observed compared to the results obtained from investigations upon the influence of ginger on seed germination.

This research states that the ginger stimulates seed germination and has both preventive and curative action against the pathogen *Alternaria alternata*.

REFERENCES

Ahmad, M. S., Qureshi, A. H. (2017). Antifungal activity of *Zingiber officinale* oil against plant pathogenic fungi isolated from solanaceous vegetable fruits. *Asian Journal of Pharmacy and Pharmacology*, 3: 121-124.

Baicu, T. (1968). Culegere de metode pentru testarea produselor fitofarmaceutice (in Romanian).

Constantinescu O. (1974). Metode şi tehnici în micologie. Ed. Ceres, Bucureşti (in Romanian).

Ebrahimzadeh Attari, V., Ostadrahimi, A., Asghari Jafarabadi, M., Sajjad Mehralizadeh, S. & Mahluji, S. (2016). Changes of serum adipocytokines and body weight following *Zingiber officinale* supplementation in obese women: a RCT. *Eur J Nutr* 55: 2129-2136.

Fawzi, E. M., Khalil, A. A. & Afifi, A. F. (2009). Antifungal effect of some plant extracts on *Alternaria alternata* and *Fusarium oxysporu*m. *Afr. J. Biotechnol*., 8: 2590-2597.

Feng, W. & Zheng, X. (2007). Essential oils to control *Alternaria alternata in vitro* and *in vivo*. *Food Control*, 18, 9: 1126-1130.

Feng, T., Su, J., Zhi-Hui Ding, Z.-H., Zheng, Y.-T., Li, Y., Leng, Y. & Liu, J.-K. (2011). Chemical constituents and their bioactivities of "Tongling White Ginger" (*Zingiber officinale*). *J Agric Food Chem.* 59(21):11690-5.

Hulea, A. (1969). Ghid pentru laboratoarele de micologie şi bacteriologie, Ed Agrosilvica, Bucureşti (in Romanian).

Iacomi, Vasilescu, B., Avenot, H., Bataille-Simoneau, N., Laurent, E., Guenard, M. & P., Simoneau (2004). In vitro fungicide sensitivity of *Alternaria species* pathogenic to crucifers and identification of *Alternaria brassicicola* field isolates highly resistant to both dicarboximides and phenylpyrroles. *Crop Protection*, 21:484-488.

Imamović, B., Komlen, V., Gavrić, T., Sunulahpašić, A., Lalević, B., Hamidović, S. (2021). Antimicrobial activity of ginger (*Zingiber officinale*) and rosemary (*Rosmarinus officinalis*) essential oils. *Agriculture and Forestry* 67 (1): 231-238.

Kapsa, J. (2009). Effectiveness of some fungicides in control of *Alternaria alternata* and *Alternaria solani*. *Proceedings of the Eleventh EuroBlight Workshop*, Hamar, Norway, 28-31 October 2008-2009: 127-134.

Karuppiah, P. & Rajaram, S. (2012). Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. *Asian Pac J Trop Biomed.* 2: 597-601.

Khandouzi, N, Shidfar, F, Rajab, A, Rahideh, T, Hosseini, P. & Taheri, MM. (2015). The Effects of Ginger on Fasting Blood Sugar, Hemoglobin A1c, Apolipoprotein B, Apolipoprotein AI and Malondialdehyde in Type 2 Diabetic Patients. *Iranian journal of pharmaceutical research* 14(1):131-140.

Malone, J.P. & Muskett, A.E. (1964). Seed-borne fungi. Proc. ISTA, no.29, pp.176.

Manole, M. S., Ciocoiu, E. (2011). Manual de Microbiologie. Ediţia a II-a revizuită şi adăugită. Ed. Alpha MDN, Bucureşti (in Romanian).

Osman, E. H. B., Algam, S. A. E., Osman, T. M. T., Ali, M. E., Abbo, A. S. H. & Elhassan, S. M. (2016). Antifungal effect of camel urine and ginger water extract against *Alternaria alternata* the causal agent of early blight disease of tomato in vitro. *International Journal of Agriculture, Forestry and Plantation*, 2: 261-69.

Park, M., Bae, J., Lee, D,-S. (2008). Antibacterial activity of gingerol and gingerol isolated from ginger rhizome against periodontal bacteria. *Phytother Res* 22(11): 1446-9.

Poltronieri, J., Becceneri, A.B., Fuzer, A.M., Cesar, J.C., Martin, A.C.B.M., Vieira, P.C., Pouliot, N. & Cominetti, M.R. (2014) [6]-gingerol as a Cancer Chemopreventive Agent: A Review of Its Activity on Different Steps of the Metastatic Process. *Mini-Rev. Med. Chem.* 14, 313-321.

Rădulescu, E., Negru, Al. (1966). Îndrumător pentru determinarea bolilor şi dăunătorilor la seminţe. Edit. Agro-Silvică, Bucureşti (in Romanian).

Sharma, N., Tiwari, R. (2013). Biological effects of ginger (Z*ingiber officinale* Roscoe.) essential oil on *Alternaria alternata* (fr.) Keissl. *International Journal of Recent Scientific Research*, 4, 827-831.

Sharma, N., Tiwari, R. & Srivastava, M. P. (2013). A Remedy for Biodeterioration: *Zingiber officinale* Roscoe. Oil. *Journal of Ornamental & Horticultural Plants* 3 (3): 191-201.

Tozlu, E., Tekiner, N., Kotan, R. & Ortucu, S. (2018). Investigation on the biological control of *Alternaria alternata*. *Indian Journal of Agricultural Sciences* 88 (8): 1241-7.

Wang, S., Zhang, C., Yang, G. & Yang, Y. (2014). Biological properties of 6-gingerol: a brief review. *Nat. Prod. Commun*. 9: 1027-1030.

Karaman, G. (2022): Research of two species of genus Niphargus Schiödte 1849 (fam. Niphargidae)(contribution to the knowledge of the Amphipoda 325). Agriculture and Forestry, 68 (1): 41-70. doi:10.17707/AgricultForest.68.1.03

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RESEARCH OF TWO SPECIES OF GENUS *NIPHARGUS* SCHIÖDTE 1849 (FAM. NIPHARGIDAE) (CONTRIBUTION TO THE KNOWLEDGE OF THE AMPHIPODA 325)

SUMMARY

Two subterranean species of genus *Niphargus* Schiödte, 1849 (Amphipoda, fam. Niphargidae) are studied: *Niphargus angelieri* Ruffo 1954 known from SW France [locus typicus: Le Boulou, Pyrenees Orientales, France] is mentioned, and new subspecies, *Niphargus angelieri ariegei*, ssp. nov. from Cave Tuc d`Audoubert, near Montesquieu-Avantes, (Dept. Ariege, Pyrenee-Orientales, France) is discovered and described.

The species *Niphargus* (*Niphargus*) *podpecanus* S. Karaman 1952, known from several localities in Slovenia [locus typicus: Podpeška Jama cave, Slovenia] is mentioned. The specimens from Źeljnske jame Cave, Źeljne, Kočevje (Slovenia), separated from *N. podpecanus* as a new species by Delić et al. (2017) based on the molecular/genetic data only, *Niphargus gottscheeanensis* Delić et al. 2017, are here morphologically described and drawn. Taxonomical and morphological characters and value of these taxa are discussed.

INTRODUCTION

Genus *Niphargus* Schiödte, 1849-Complex (fam. Niphargidae) settled subterranean waters from N. Spain and England till Iran and Caucasus with over 300 different known and numerous still undiscovered new taxa. The great diversity of morphological characters of *Niphargus*-Complex indicated that settlements of subterranean waters by *Niphargus*- Complex occur several times from Tertiary till today, followed by very successful morphological and other modification and adaptation to the various subterranean environments, from primitive forms with ability to bent the body into a ball (*K. parapupetta* Karaman G. 1984) till slender forms with ability to pass between grains of sand (*N. kragujevensis* Karaman S. 1950). This process is still active today, observed by high morphological diversity and morphological variations within specimens of one locality, as well as between various populations of one species and presence

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of transitive populations between different populations still in process of reproductive isolation.

For this reason there are wide discordance about validity and delimitation of various taxa, recognition of subspecies or transitive population based of morphological and molecular-genetic research. We tried to show here some of these dilemma, where only multiple approaches to this problem are needed.

MATERIAL AND METHODS

The studied material was preserved in the 70% ethanol. The specimens were dissected using a WILD M20 microscope and drawn using camera lucida attachment. Body-parts were temporarily submersed in the mixture of glycerin and water for study and drawing by camera lucida; later transferred to Liquid of Faure as permanent slides. All illustrations were inked manually.

Some morphological terminology and setal formulae follow G. Karaman`s terminology (Karaman, G. 1969; 2012) for the last mandibular palpus article [A= A-setae on outer face; B= B-setae on inner face; C= additional C-setae on outer face; D= lateral marginal D-setae; E= distal long E-setae], and for propodus of gnathopods 1 and 2 [S= corner S-spine; L= lateral slender serrate L-spines; M= facial corner M-setae; R= subcorner R-spine on inner face].

Terms “setae” and “spines” are used based on shape, not origin. Our studies were based on the external morphology, ecology and zoogeography.

TAXONOMICAL PART

Family NIPHARGIDAE

NIPHARGUS ANGELIERI ANGELIERI Ruffo 1954

Niphargus skopljensis angelieri Ruffo 1954: 673, figs. II and III; ; Balazuc 1957: 76; Barnard & Barnard 1983: 695;
Niphargus angelieri G. Karaman & Ruffo 1986: 522; Ginet 1995/6: 53, figs. II-III; Ferreira et al. 2007: 589 (no localities); Bréhier & Jaume 2009: 18.

LOCUS TYPICUS: Le Boulou, Pyrenees Orientales, France.

LOCALITIES CITED:
Ruffo 1954: cited this species for 4 localities of Pyrenees orientales in France: Le Boulou; Pont de Boulou; Baillaurie, Banyuls-sur Mer; La Raillere, Amelie-les-Bains;
Ginet 1996: Pyrenee Orientales;
Bréhier & Jaume 2009: French Mediterranean coast: cave, “Grotte des Fées de Leucate”.

DISTRIBUTION:
Western part of Pyrenees orientales near Mediterranean coast (France), in interstitial waters and caves.

REMARKS.
N. angelieri angelieri, although mentioned by various authors, remains only partially described, and some important taxonomical characters are unknown. The specimens from Cave Tuc d`Audoubert, locality nearly 170 km W of the Mediterranean Sea coast, differing from typical *N. angelieri* by several characters, is described and figured here as a new subspecies, *N. angelieri ariegei*, ssp. nov.

NIPHARGUS ANGELIERI ARIEGEI, ssp. nov.
Figures 1-5

MATERIAL EXAMINED:
S-7413= Cave Tuc d`Audoubert, near Montesquieu- Avantes (Dept. Ariège, Pyrenee-Orientales, France), nearly 10 exp (leg. G.M.).

DIAGNOSIS (based on female):
Adult females up to 3 mm, urosomal segment 1 with seta, urosomal segment 2 with spines. Coxae short, coxa 1 with subrounded ventroanterior corner, coxa 4 unlobed; epimeral plates 2-3 angular, with subventral spines. Antenna 1 slender, peduncular articles 1-3 progressively shorter, accessory flagellum 2-articulate, almost as long as peduncular article 3; antenna 2 slender, flagellum longer than last peduncular article. Mandibular palpus article 3 with partially reduced number of D and A-setae, B-setae absent.

Maxilla 1 inner plate with 2 setae, 6 spines of outer plate with 3-5 teeth each, one spine (inner) spine with 7 lateral teeth, palpus not reaching tip of outer plate spines, with 3-4 distal setae. Maxilliped inner plate exceeding outer tip of palpus article 1, provided with 3 spines; outer plate not exceeding tip of palpus article 2, nail of palpus article 4 only slightly shorter than pedestal.

Gnathopods 1-2 relatively small, article 5 rather elongated; propodus trapezoid, inclined, with convex corner, L-spines sitting laterally of S-spine, one corner facial M-seta, dactylus with one median seta at outer margin. Dactylus of pereopods 3-7 with one slender spine-like seta at inner margin. Pereopods 5-7 progressively longer and stronger, article 2 dilated, with ventroposterior lobe. Pleopods almost naked, with 2 retinacula. Uropod 1 peduncle with dorsointernal and dorsoexternal row of spines, inner ramus off both uropods with longer inner ramus than outer one, distal spines short. Uropod 3 short and strong, distal article of outer ramus short, first article along both margins with bunches of spines, at mesial margin mixed with single plumose setae. Telson incised nearly 2/3 of telson-length, lobes with distomesial narrowed tip and distal 2 short spines sitting at distolateral side; pair of short plumose setae attaches on each lobe lateromedially.

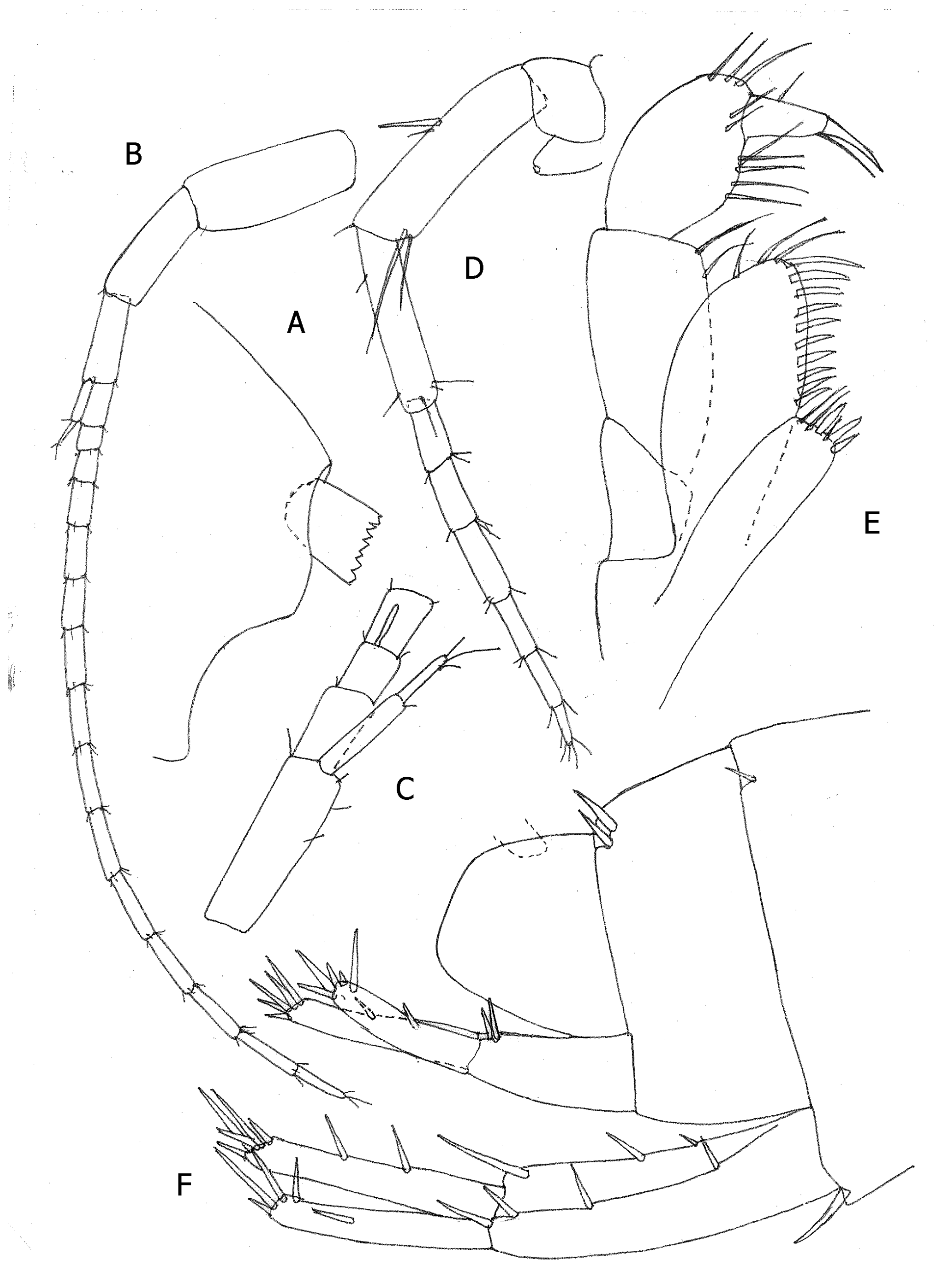

Fig. 1. *Niphargus angelieri ariegei*, ssp. nov., Cave Tuc d`Audoubert, near Montesquieu-Avantes, female 3.0 mm: A= head; B= antenna 1; C= accessory flagellum; D= antenna 2; E= maxilliped; F= urosome with uropods 1-2.

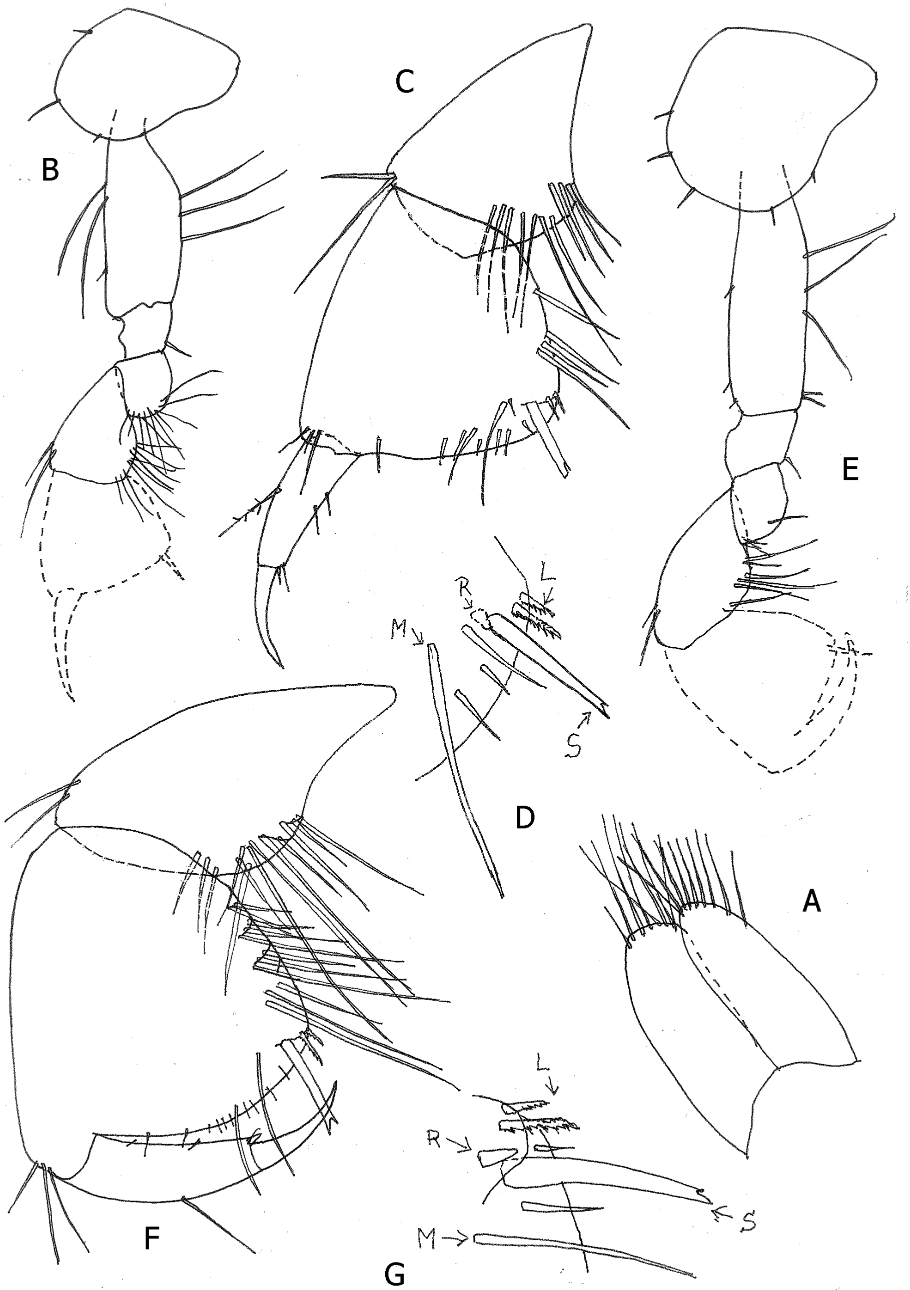

Fig. 2. *Niphargus angelieri ariegei*, ssp. nov., Cave Tuc d`Audoubert, near Montesquieu-Avantes, female 3.0 mm: A= maxilla 2; B-C= gnathopod 1, outer face; D= distal corner of gnathopod 1-propodus, outer face [S= corner S-spine; L= lateral L-spines; M= corner facial M-seta; R= subcorner R-spine, inner face]; E-F= gnathopod 2, outer face; G= distal corner of gnathopod 2-propodus, outer face [S= corner S-spine; L= lateral L-spines; M= corner facial M-seta; R= subcorner R-spine, inner face].

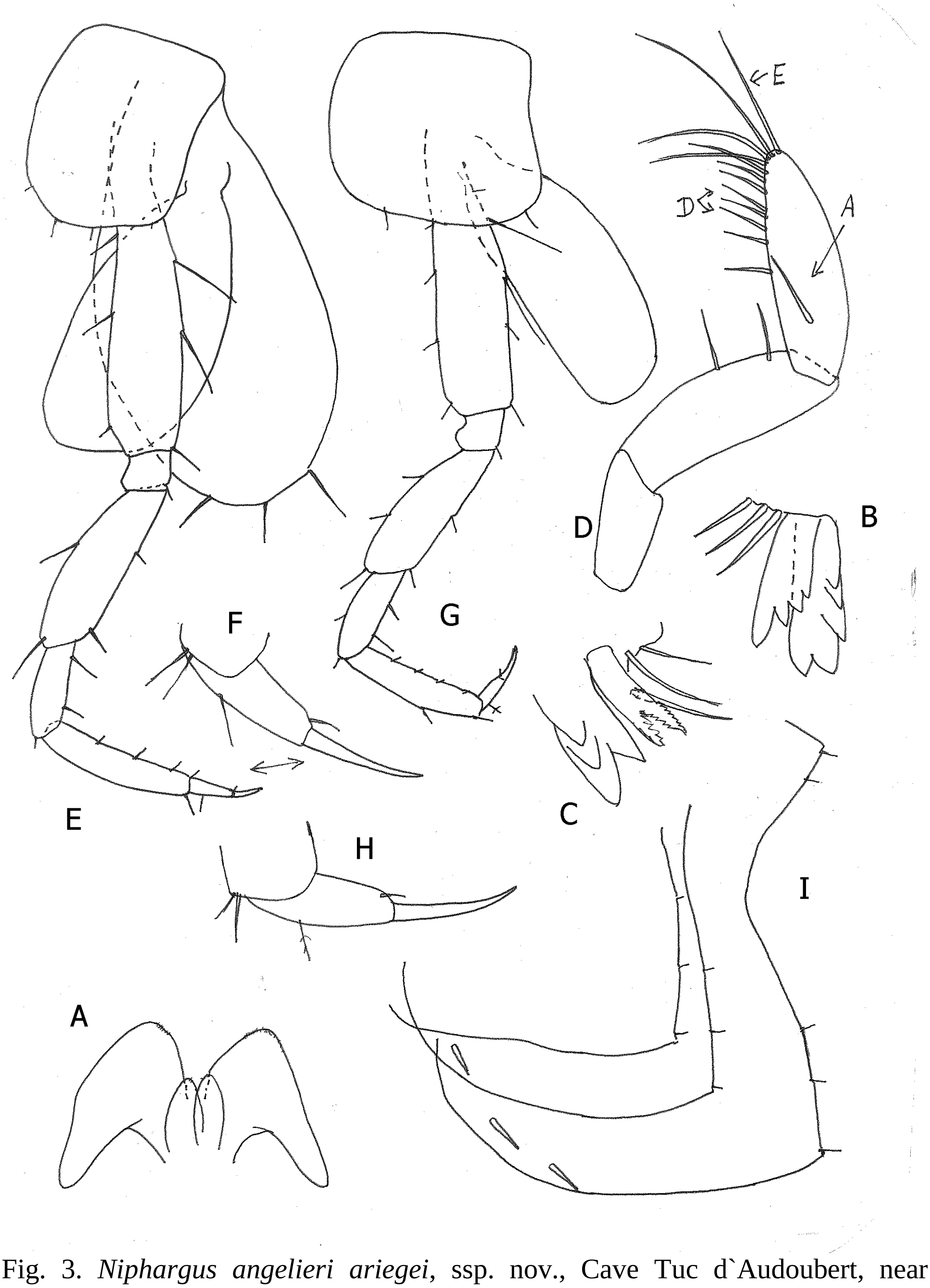

Fig. 3. *Niphargus angelieri ariegei*, ssp. nov., Cave Tuc d`Audoubert, near Montesquieu-Avantes, female 3.0 mm: A= labium; B= left incisor with lacinia mobilis and rakers; C= right incisor with lacinia mobilis and rakers; D= mandibular palpus, outer face [D= marginal D-setae; E= distal E-setae; A= facial A-seta]; E-F= pereopod 3; G-H= pereopod 4; I= epimeral plates 1-3.

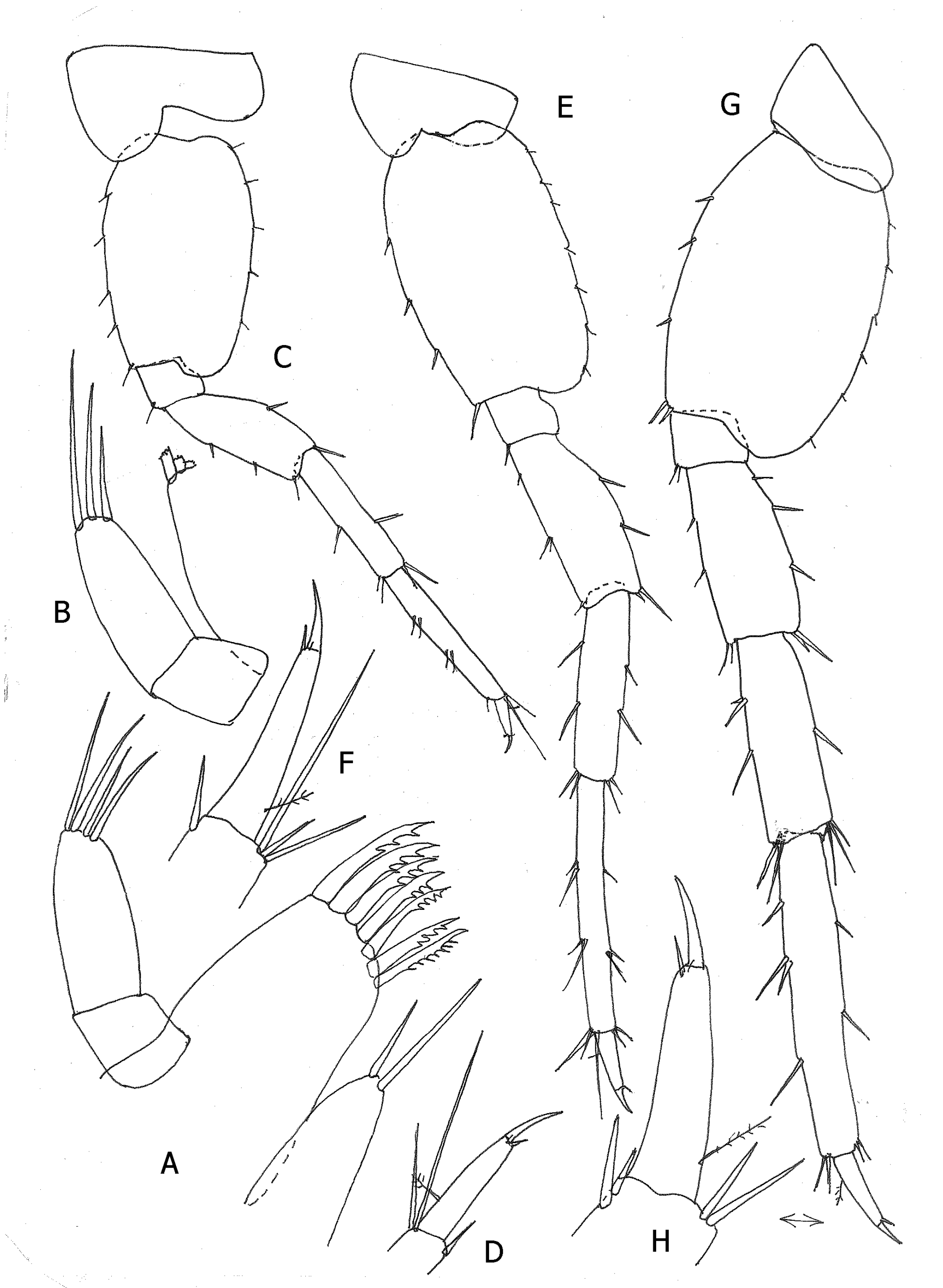

Fig. 4. *Niphargus angelieri ariegei*, ssp. nov., Cave Tuc d`Audoubert, near Montesquieu-Avantes, female 3.0 mm: A-B= maxilla 1; C-D= pereopod 5; E-F= pereopod 6; G-H= pereopod 7

DESCRIPTION.

Female ovig. 3.0 mm with setose oostegites: Body moderately slender, metasomal segments 1-3 with 3-4 dorsoposterior short marginal setae (fig. 3 I); urosomal segment 1 with one dorsolateral seta on each side; urosomal segment 2 on each dorsolateral side with 2 spines; urosomal segment 3 naked (fig. 1F). Epimeral plates distinctly angular: epimeral plate 1 with vestigial ventroposterior tooth and posterior convex margin bearing 2 setae, ventral margin naked, slightly concave in the middle. Epimeral plate 2 with distinct ventroposterior corner and poorly convex posterior margin almost straight in distal part and provided with 1-2 short setae, ventral margin convex, with one submarginal spine (fig. 3 I); epimeral plate 3 with marked ventroposterior tooth, convex posterior margin bearing 3 setae, ventral margin convex, with 2 subventral spines.

Head with very short rostrum, lateral cephalic lobes shallow, obtuse, ventroanterior excavation developed, eyes absent (fig. 1A).

Antenna 1 slightly exceeding half of body; peduncle relatively slender, with 3 articles (ratio: 48:35:25) almost naked (fig. 1B); main flagellum 15 slender scarcely setose articles (most of them with one aesthetasc exceeding half of article itself (fig. 1C). Accessory flagellum 2-articulated, almost as long as last peduncular article (ratio: 46:50), distal article elongated (fig. 1C).

Antenna 2 relatively slender, peduncular article 3 short; article 4 at dorsal margin with one median spine and short seta, distoventral tip with 2 long setae, dorsal distal tip with one short seta (fig. 1D); article 5 more slender and shorter than article 4 (ratio: 54:65), with single short marginal and distal setae. Flagellum longer than last peduncular article, consisting of 6 stronger articles provided with several short distal setae each. Antennal gland cone short (fig. 1D).

Mouthparts basic. Labrum slightly broader than long, convex distally (fig. 5A). Labium broader than long, outer lobes subrounded distally, inner lobes small but well developed (fig. 3A).

Mandible with triturative molar. Left mandible: incisor with 5 teeth, lacinia mobilis with 4 teeth and 3 rakers (fig. 3B). Right mandible: incisor with 4 teeth, lacinia mobilis bifurcate and serrate, accompanied by 3 rakers (fig. 3C). Palpus mandibulae with 3 articles: first article short, naked; second article with 2 setae; third article poorly subfalciform, with 7 D-setae and 5 distal E-setae, on outer face with one facial A-seta, B-setae on inner face absent (fig. 3D).

Maxilla 1: inner plate narrowed, with 2 distal setae; outer plate with 7 spines [5 spines with 3-5 teeth, inner spine with up to 7 teeth (fig. 4A)]; palpus short, 2-articulated, not reaching distal tip of outer plate spines, bearing 3-4 unequal setae (figs. 4A, B).

Maxilla 2 longer than broad, inner plate poorly smaller than outer one, both plates with distal setae only (fig. 2A).

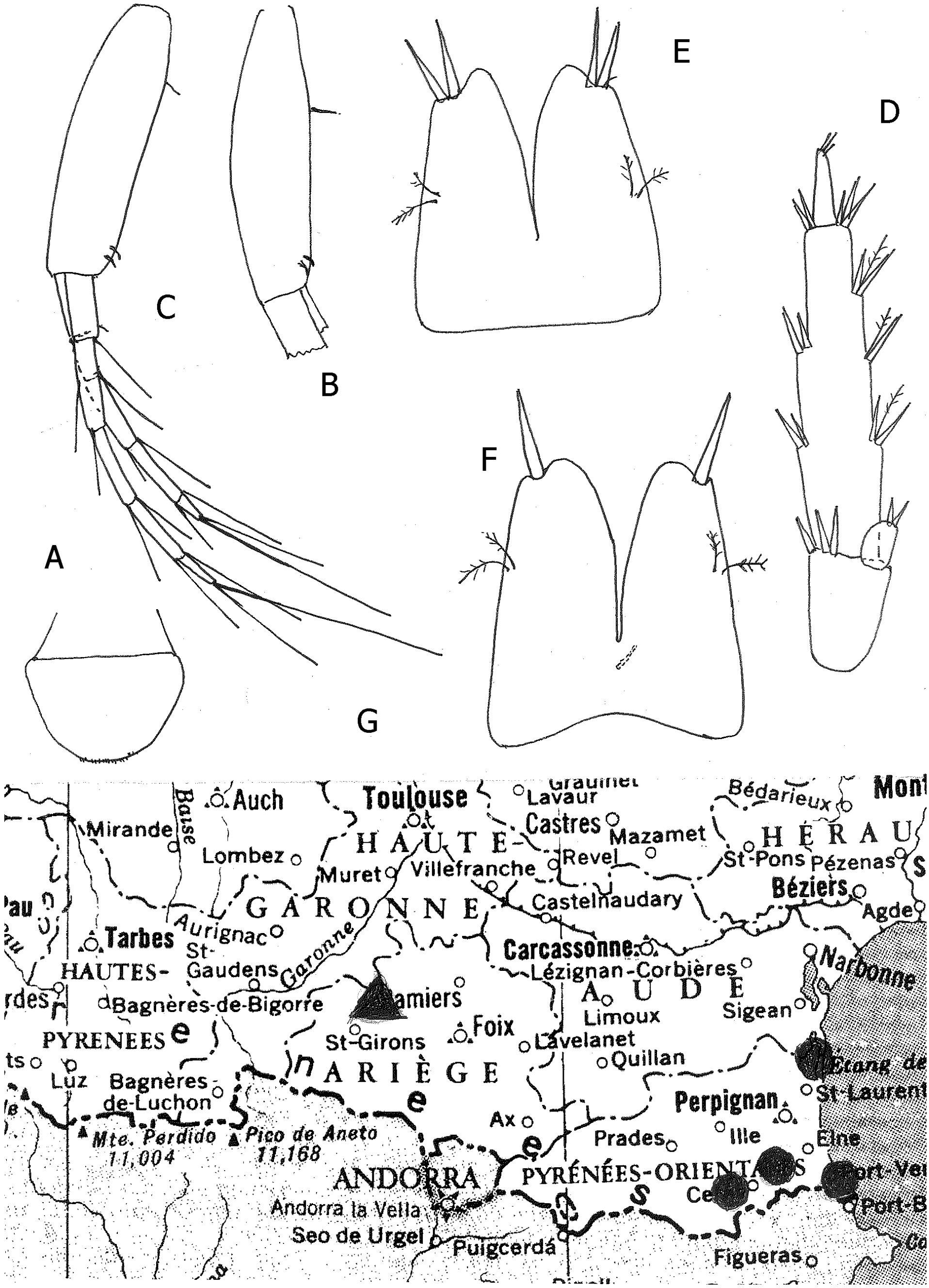

Fig. 5. *Niphargus angelieri ariegei*, ssp. nov., Cave Tuc d`Audoubert, near Montesquieu--Avantes, female 3.0 mm: A= labrum; B= pleopod 1-peduncle; C= pleopod 3-peduncle; D= uropod 3; E= telson; F= telson, female 2.9 mm; G= Distribution of *Niphargus angelieri* in France: dots= *N. angelieri angelieri*; triangle= *N. angelieri ariegei*

Maxilliped: inner plate slightly exceeding outer tip of palpus article 1, with 3 distal spines and 3-4 setae; outer plate long, reaching tip of palpus article 2 only, along inner (mesial) margin with nearly 9 strong spines and 3 long distal setae (fig. 1E); palpus article 2 at inner (mesial) margin with row of setae; article 3 with 3 distal and 3-4 distolateral setae, at inner (mesial) margin with 5 setae; article 4 nail slightly shorter than pedestal, outer margin with median seta, inner margin with one seta near basis of the nail (fig. 1E).

Coxae relatively short. Coxa 1 broader than long (ratio: 44:37), broadly subrounded ventroanterior corner, bearing 3 marginal setae (fig. 2B). Coxa 2 nearly as long as broad, convex ventral margin with 4 setae (fig. 2E). Coxa 3 longer than broad (ratio: 58:48), with 3-4 marginal setae (fig. 3E). Coxa 4 broader than long (ratio: 59:55), with 2 ventral setae and without ventroposterior lobe (fig. 3G).

Coxae 5-7 shallow. Coxa 5 is shorter than coxa 4, broader than long (ratio: 55:33), ventroanterior lobe subrounded, not produced (fig. 4C). Coxa 6 smaller than 5, broader than long (ratio: 48:28) (fig. 4E). Coxa 7 shorter than 6, entire, broader than long (ratio: 44:23), tapering posteriorly (fig. 4G).

Gnathopods 1-2 relatively small, propodus not larger than corresponding coxa. Gnathopod 1 slightly smaller than 2, article 2 with 2 long median setae at anterior margin and 3 long median setae at posterior margin (fig. 2B); article 3 with one posterior seta; article 4 with posterior bunch of setae; article 5 nearly as long as propodus, with 2 distoanterior longer setae, at posterior margin with 2 rows of setae. Propodus trapezoid, hardly longer than broad (ratio: 74:69), posterior margin with 2 transverse rows of long setae, anterior margin with 3 distal setae (fig. 2C). Palm convex, inclined nearly 2/5 of propodus-length, defined on outer face by corner S-spine accompanied laterally by 2 serrate L-spines and one corner facial M-seta (fig. 2D), on inner face by one small subcorner R-spine. Dactylus reaching posterior margin of propodus, one median seta at outer margin and 3 shorter setae along inner margin (fig. 2C).

Gnathopod 2: article 2 with 3-4 short anterior marginal setae, posterior margin with 3 long median setae and bunch of distoposterior short setae (fig. 2E); article 3 with one distoposterior seta; article 4 with several posterior marginal short setae. Article 5 nearly as long as propodus, with 2 distoanterior setae, posterior margin with numerous long setae (fig. 2E). Propodus trapezoid, longer than broad (ratio: 94:81), with 3 distoanterior setae, posterior margin with 4 transverse rows of long setae (fig. 2F). Palm convex, inclined nearly 1/3 of propodus-length, defined on outer face by corner S-spine accompanied laterally by 2 serrate L-spines and one corner facial M-seta (fig. 2G), on inner face by one subcorner S-spine. Dactylus reaching posterior margin of propodus, one median seta at outer margin and 4 short setae at inner margin (fig. 2F).

Pereopods 3-4 moderately slender. Pereopod 3 rather longer than 4, article 2 with 3 shorter anterior marginal setae, 2 long median and one short distal seta at posterior margin (fig. 3E); article 3 with short distoposterior seta. Articles 4-5 of different length (ratio: 53:28:44), along both margins with scarce number of short setae or spine-like setae; article 6 at posterior margin with row of 4 short setae. Dactylus remarkably shorter than article 6 (ratio: 22:44), inner margin with one longer strong seta, outer margin with one median plumose seta (fig. 3F); nail rather shorter than pedestal (ratio: 43:57).

Pereopod 4: article 2 anterior margin with 4 short setae, posterior margin with 2 median long setae and 3 short setae; article 3 with distoposterior short seta. Articles 4-6 of different length (ratio: 45:27:40): articles 4-5 with several single short setae at both margins; article 6 at posterior margin with 5 short setae (fig. 3G). Dactylus shorter than article 6 (ratio: 21:40), one stronger seta at inner margin (fig. 3H), outer margin with one median plumose seta; nail shorter than pedestal (ratio: 37:45).

Pereopods 5-7 moderately slender. Pereopod 5 remarkably shorter than pereopods 6 and 7, article 2 dilated, longer than broad (ratio: 68:43), slightly tapering ventrally, anterior convex margin with 6 strong setae, posterior convex margin with 4-5 short setae, ventroposterior lobe developed (fig. 4C). Articles 4-6 of different length (ratio: 40:42:48); articles 4-5 with single short setae or spine-like setae at both margins; article 6 anterior margin with 3 groups of short setae and distal seta longer than dactylus. Article 2 longer than article 6 (68:48). Dactylus much shorter than article 6 (ratio: 15:48), one stronger seta at inner margin and one median plumose seta at outer margin (fig. 4D); nail shorter than pedestal (ratio: 19:34).

Pereopod 6: article 2 larger than that of pereopod 5, almost not tapering ventrally, longer than broad (ratio: 80:48), anterior convex margin with 4 spine-like setae, posterior margin almost straight medially, bearing 7-8 short setae, ventroposterior lobe shallow (fig. 4E). Articles 4-6 of different length (ratio: 51:55:72); both margins with single or paired spine-like setae or slender spines. Article 2 longer than article 6 (ratio: 80:72). Dactylus shorter than article 6 (ratio: 19:72), strong seta at inner margin and median plumose seta at outer margin; nail shorter than pedestal (ratio: 20:56) (fig. 4F).

Pereopod 7 longer than pereopod 6, article 2 hardly ovoid, longer than broad (ratio: 87:57), anterior convex margin with 4-5 slender spines, posterior convex margin with 6 short setae, ventroposterior lobe developed (fig. 4G). Articles 4-6 of different length (ratio: 51:59:92), both margins with single or paired slender spines up to as long as diameter of articles themselves. Article 2 shorter than article 6 (ratio: 87:92). Dactylus remarkably shorter than article 6 (ratio: 30:92), one slender spine-like seta at inner margin and one median plumose seta at outer margin (fig. 4H); nail shorter than pedestal (ratio: 31:65).

Pleopods with 2 retinacula, external rami with 6 articles, internal rami with 5 articles. Peduncle of pleopod 2 naked, that of pleopods 1 and 3 with one proximal posterior seta (fig. 5B, C).

Uropods 1-3 stout, with relatively short spines. Uropod 1 with dorsoexternal and dorsointernal row of spines (fig. 1F). Inner ramus (without distal spines) shorter than peduncle, bearing 2 lateral and 5 strong, relatively short distal spines; outer ramus rather shorter than inner one, with one lateral and 4 distal strong spines (fig. 1F).

Uropod 2 peduncle with distal spines; inner ramus with 2 lateral and 5 distal spines; outer ramus remarkably shorter than inner one, with 5 distal spines (fig. 1F).

Uropod 3 short, peduncle rather longer than broad, with 3-4 distal spines; inner ramus scale-like, shorter than peduncle, bearing 2 distal spines. Outer ramus 2-articulated: first article along outer margin with 3 groups of spines, along inner (mesial) margin with 4 groups of strong spines accompanied by one short plumose seta (fig. 5D); second article short, not exceeding diameter of first article, bearing 3 short distolateral simple setae.

Telson short, nearly as long as broad, incised nearly 2/3 of telson-length (fig. 5E); tapering distally; each lobe with distomesial subrounded tip, and 2 distal spines sitting at distolateral margin; the longest spines reaching 2/5 of telson-length only. A pair of short plumose setae attached medially at outer margin of each lobe.

Gills moderately large, not exceeding distal margin of corresponding article 2 (fig. 3E, G).

Oostegites large, with marginal setae (fig. 3E).

MALE unknown; we suppose that it will be similar to females.

VARIABILITY.

All ovigerous females up to 3 mm are with setose oostegites (fig. 3E). Telson always with distolateral position of 1-2 distal short spines (fig. 5E, F), lateral and facial spines absent; a pair of median plumose setae short; inner plate of maxilliped with 2-3 distal spines and single setae. Maxilla 1 palpus not reaching distal tip of outer plate spines, bearing 3-4 distal setae (fig. 4A, B).

The juvenile specimens of 2.0 mm show the same main taxonomical characters as adult females (uropod 3, shape of telson, maxilliped, maxilla 1, etc.).

REMARKS AND AFFINITIES.

Our specimens from Tuc d` Audoubert are very similar to *Niphargus angelieri* Ruffo 1954 known from several localities of Pyrenees Orientale, in the subterranean waters [Le Boulou; Pont du Boulou; Baillaurie (Banyuls sur Mer); La Raillere (Amelie-les- Bains] and cave (Grotte des Fées de Leucate)] and we suspected that our taxon can be identical with *N. angelieri*. Unfortunately, there are no further taxonomical data of specimens of *N. angelieri* cited from other localities, except those quoted by Ruffo from locus typicus.

One more detailed analyze of known taxonomic characters, suggested that, based on present knowledge of *N. angelieri*, specimens from Tuc d`Audoubert are not identical with ssp. *angelieri,* but represent a distinct new taxon, *N. angelieri ariegei*, ssp. new.

The subspecies *N. angelieri ariegei* differs from ssp. *angelieri* (based of figures and description of Ruffo) by:

- Maxilla 1 palpus 3-4 setae (3 in *angelieri*); Maxilliped inner plate not reaching half of second palpus article 2 (distinctly reaching second palpus article in *angelieri*); outer plate not exceeding tip of palpus article 2 (distinctly exceeding in *angelieri*); nail of article 4 almost reaching pedestal, with long seta at inner margin near basis of the nail (shorter in *angelieri*);
- Coxa 3 is not broader than long (broader than long in *angelieri*);
- Pereopod 7 article 2 more elongated and with longer nail of dactylus;
- Epimeral plates 2-3 with distinctly pointed ventroposterior corner and provided with subventral spines (obtuse, without spines in *angelieri*);
- Pleopods with 2 retinacula (undescribed in *angelieri*);
- Uropods 1-2: both rami with short distal spines (very long in *angelieri*); Uropod 3: outer margin of outer ramus with 3 bunches of short spines (single simple setae only in *angelieri*);
- Telson: lobes with distal finger and 1-2 short distolateral spines reaching 2/5 of telson length (lobes subrounded distally, with 3 slender distal spines (the longer spines reaching almost half of telson-length in *angelieri*); Pair of lateral plumose setae of telson-lobes short (one of these plumose setae very long in *angelieri*);

The sex of described *N. angelieri* is not mentioned.

Although some taxonomic differences we can theoretically attributed to the size or different sex, the most of them are more stable (mouthparts, telson, geographical distance). But we cannot exclude the possibility that our specimens can be within the unknown limits of morphological variety of still poorly known *N. angelieri*.

NIPHARGUS (NIPHARGUS) PODPECANUS **S. Karaman 1952**

Niphargus (Stygoniphargus) stygius podpecanus S. Karaman 1952: 12, pl. VI, figs. 24-28;

Niphargus stygius podpecanus G. Karaman 1972: 6; G. Karaman 1974: 26; G. Karaman & Ruffo 1986: 532.

Niphargus podpecanus Delić et al. 2017: 6; Primate et al. 2021: 3;

LOCUS TYPICUS: Podpeška Jama cave, Slovenia.

LOCALITIES CITED: SLOVENIA:

S. Karaman, 1952: Podpeška Jama Cave; “Wasserloch”(=swamp) near Kočevje; well in Kočevje;

G. Karaman 1974: loc. typ.; Kočevje;

Delić et al. 2017: Podpeška jama Cave, Podpeč, Dobrepolje; Slugova jama Cave, Golobinjek, Dolenjske toplice; spring near Venska vas village, Kočevje; spring near Obrh, Mirtoviči, Osilnica; Krška jama Cave, Gradiček, Krška vas village; Željnske jame Cave, Željne, Kočevje; Črničkova jama Cave, Stavča vas village, Žužemberk; Lučka jama Cave, Luče, Grosuplje; Jama v Peklu Cave, Rajndol, Kočevje; spring near Obrh, Mirtoviči, Osilnica.

Premate et al. 2021: Podpeška jama Cave, with *N. longiflagellum* S. Karaman 1950 and *N. pachytelson* Sket 1960.

REMARKS.

Niphargus (Niphargus) podpecanus belongs to *N. stygius*-Complex. *N. podpecanus* from type-locality (Podpeška jama Cave, Slovenia) is rather similar to *N. stygius* (Schiödte 1847) [loc. typ. Postojna Cave, Slovenia] but differs from later by various morphological characters (presence of facial spines on telson, presence of plumose setae on peduncle of pleopods, gnathopods 1-2, uropod 1 in males, etc).S. Karaman (1952) cited this species from several localities: Podpeška jama Cave; well in Kočevje; "Wasserloch" (= swamp} near Kočevje. He mentioned (1952: 14) that specimens from one of four his samples from Podpeška jama Cave were slightly different (more inclined palm of gnathopods, rather shorter inner ramus of uropod 1, rather shorter distal article of uropod 3 outer ramus, slightly more angular epimeral plates, and with weak facial spine on telson or absence of this spine), considering that this sample can be maybe rather different form between *valvasor*i and *podpecanus*. S. Karaman proposed the possibility that in different parts of the same subterranean water can develop or preserve different forms morphologically different.Delić et al. (2017) have split *Niphargus podpecanus* sensu S. Karaman into 3 different species based on partially genetic/molecular analyze, without detailed morphological data of taxonomical characters and differences. One of them, *Niphargus gottscheeanensis* Delić et al. 2017 [locus typicus: Źeljnske jame Cave] was cited also for Podpeška jama Cave, mentioning that there are not morphological differences between *podpecanus* and *gottscheeanensis*. Unfortunately they have not redescribed and drawn neither specimens from Podpeška jama nor these from Źeljnske jame Caves, to support this conclusion.

1. There are no doubt that within the same subterranean waters (cave system), the ecological, physical, chemical and other conditions can be rather different, what can cause some morphological or partially known genetic/molecular differences (or both of them).

2. Evidently not every established molecular/genetic difference of specimens presented in one sample, represent automatically a new species, especially when both nominated new species have identical morphological characters.

The similar problem I established in epigean populations of amphipod *Gammarus balcanicus*- Complex in several torrents and rivers (G. Karaman, 1977: 51), but neither attention nor reaction to this problem was later made.

Within one water stream in the source zone, some morphological characters of *G. balcanicus* s. l. are different that these in specimens some kilometers far downstreams (in males and females), what will be enough to consider the presence of two different species, because there are no transitive specimens in contact zone. But, detailed morphological analyze of specimens further downstreams show presence of specimens with transitive morphological characters between both forms, indicating that they are not species but only different forms of the same species (G. Karaman 1977: 51). In some torrents we found also 3 different populations of the same species (*G. balcanicus* s. l.) and always with transitive specimens in transitive zone.

I suppose that the similar situation is within some subterranean populations of single *Niphargus* species also. The recent oversplitting of single known species into several distinct "cryptic" new species each, based on molecular/genetic differences only, although with identical morphological characters, must be reexamined and confirmed through further detailed study of other genetic/molecular and morphological data. There are probability that some of these "cryptic species" can be various varieties and different forms of one species. Are there all numerous new cryptic species reproductively isolated? Absence of cited subspecies or transitive populations among so many "nominated cryptic species" indicated necessity of reexamination of all these split taxa.

As *N. podpecanus* s. str. is only partially described and figured, and split taxon *N. gottscheeanensis* was not described morphologically and figured, we described and figured *N. gottscheeanensis*, to help further study of its taxonomical status regarding *N. podpecanus* and entire *N. stygius*-Complex.

NIPHARGUS (NIPHARGUS) GOTTSCHEEANENSIS Delić et al. 2017
Figures 6-12

?Niphargus (Stygoniphargus) stygius podpecanus S. Karaman 1952 (part.).
Niphargus gottscheeanensis Delić et al. 2017: 3, map 1.

MATERIAL EXAMINED: SLOVENIA

AMD/00337- Źeljnske jame Cave (S. 12), Źeljne, Kočevje, 11.5. 1993, 6 exp. intermixed with *Synurella ambulans ambulans* (F. Müller 1846) and *Niphargus longiflagellum* S. Kar. 1950 (leg. F. Gasparo);

AMD/00477- Kompoljska jama Cave (S. 25), Kompolje, Videm, 11.7.1993, several exp. (leg. J. Broder, F. Gasparo, F. Stoch).

LOCUS TYPICUS: Źeljnske jame Cave, Źeljne, Kočevje.

DIAGNOSIS

Large species, up to 21 mm long, metasomal segments with several dorsomarginal setae only; urosomal segment 1 with dorsolateral setae, urosomal segment 2 with dorsolateral spines; urosomal segment 3 naked; epimeral plates quadrate, with weakly angular ventroposterior corner and straight or slightly convex posterior margin. Head with short lateral cephalic lobes, antennae 1-2 slender, antenna 1 peduncular articles 1-3 progressively shorter, flagellum long, accessory flagellum 2-articulate. Antenna 2 slender, peduncular article 5 shorter than 4, flagellum much longer than last peduncular article.

Mandibular palpus article 1 naked, article 2 with numerous setae, article 3 as long as 2, with numerous D, E, A and B setae. Maxilla 1 inner plate with several setae, outer plate with mainly one-toothed distal spines, palpus relatively short. Maxilliped inner and outer plate short, inner plate with 4 spines. Coxae short, coxa 1 subrounded ventroanterior corner; coxa 4 without distinct ventroposterior lobe. Gnathopods 1-2 nearly as large as corresponding coxae, with trapezoid propodus remarkably inclined, L-spines sitting laterally of S-spine, inner face with one R-spine; dactylus with numerous outer marginal setae.

Pereopods 3-7 dactylus with one spine at inner margin, article 2 of pereopods 5-7 narrowed, without ventroposterior lobe. Pleopods with 2 retinacula, peduncles with additional plumose setae. Uropod 1 peduncle with dorsointernal row of setae, inner ramus in males remarkably longer than outer one. Uropod 3 elongated in males, second article long. Telson gaping, with distal, lateral and facial spines. Sexual dimorphic characters present (uropod 1, uropod 3).

DESCRIPTION (Źeljnske jame Cave). MALE 20.0 mm.

Body moderately slender, metasomal segments 1-3 with 4-5 dorsoposterior marginal setae each (fig. 9B); urosomal segment 1 with one seta on each dorsolateral side; urosomal segment 2 on each dorsolateral side with 2 spines and 1 seta, urosomal segment 3 naked (fig. 9D). Urosomal segment 1 near basis of uropod 1-peduncle with one bunch of 2 ventroposterior spine-like setae (fig. 9D).

Epimeral plates 1-3 quadrate, with marked ventroposterior corner and with convex posterior margin; plates 2-3 with 2-4 subventral spines.(fig. 9B).

Head: dorsal margin poorly convex in lateral projection, lateral cephalic lobes short and subrounded, eyes absent.

Antenna 1 reaching almost half of the body; peduncular articles 1-3 progressively shorter (ratio: 84:61:27), article 3 not reaching half of article 2; main flagellum consisting of 32 articles (most of them with one short aesthetasc) (fig. 6B); accessory flagellum short, 2-articulated (fig. 6B).

Antenna 2 moderately slender, peduncular article 5 distinctly shorter than 4 (ratio: 60:70), both articles with 4 ventral bunches of setae shorter or longer than diameter of articles themselves; flagellum slender, poorly setose, consisting of 17 articles; antennal gland cone short (fig. 6C).

Mouthparts: Labrum broader than long, convex distally (fig. 6A); labium broader than long, inner lobes short, outer lobes subrounded (fig. 10A).

Left mandible: incisor with 5 teeth, lacinia mobilis with 4 teeth, accompanied by 16 rakers (fig. 10B). Right mandible: incisor with 4 teeth, lacinia mobilis bifurcate, serrate, accompanied by 10 rakers (fig. 10C). Palpus mandibular article 1 naked; article 2 as long as article 3, bearing 15+3 setae;

article 3 with 33 D and 8 E setae (fig. 10D), on outer face with one group of 13 A-setae (fig. 10E), on inner face 4 groups of B setae (4-3-2-1) (fig. 10D); C-setae absent.

Maxilla 1: inner plate with 4 setae, outer plate with 7 spines (6 spines with 1 tooth, one spine with 3 teeth) (fig. 10F), palpus 2-articulated, short, not reaching tip of outer plate-spines, provided with 13-14 setae (fig. 10F, G).

Maxilla 2 inner plate slightly shorter than outer one, both with distal setae only (fig. 10H). Maxilliped: inner plate short, not reaching outer tip of first palpus article provided with 3-4 spines, outer plate nearly reaching half of second palpus article, with row of nearly 12 mesial pointed spines (fig. 9A), palpus article 3 with 2 outer marginal bunches of setae; article 4 at outer margin with 2 median setae, at inner margin with one seta near basis of the nail, nail shorter than pedestal.

Coxa 1 broader than long (ratio: 43:35), with narrowly subrounded ventroanterior corner (fig. 7A), bearing nearly 13 short marginal setae; coxa 2 hardly longer than broad (ratio: 45:42), with nearly 15 short marginal setae (fig. 7D); coxa 3 hardly longer than broad (ratio: 48:45), with nearly 12 short marginal setae (fig. 6D). Coxa 4 nearly as long as broad, with 12 short marginal setae (fig. 6F); ventroposterior lobe absent. Coxa 5 shorter than coxa 4, much shorter than broad (ratio: 54:33), with single marginal short setae, anterior lobe subrounded, short (fig. 8A); coxa 6 much broader than long (ratio: 45:30), with single short marginal setae (fig. 8C). Coxa 7 entire, broader than long (ratio: 42:20), with one short marginal seta (fig. 8E).

Gnathopods 1-2 moderately large, with propodus not larger than corresponding coxa (fig. 7A, D). Gnathopod 1 slightly smaller than 2, article 2 with numerous long setae along anterior and posterior margin; article 3 with distoposterior bunch of setae; article 5 shorter than propodus (ratio: 30:40), with 2 distoanterior bunches of setae and numerous posterior marginal setae (fig. 7A). Propodus trapezoid, slightly longer than broad (ratio: 90:81), with 10 transverse rows of posterior setae (fig. 7B); palm inclined slightly over half of posterior length, defined on outer face by one corner S-spine accompanied laterally by 3-4 slender toothed L-spines and 7 facial corner M-setae, on inner face by 1 short subcorner R-spine (fig. 7C); dactylus with row of setae along outer margin (1-2-2-1-1-1-1-1-1-1-2-1-2-1 or 1-1-2-1-1-1-1-1-1-2-1-1-2-1) and short setae at inner margin (fig. 7B).

Gnathopod 2: article 2 with row of numerous long setae at anterior and posterior margin; article 3 with distoposterior bunch of setae; article 5 nearly as long as propodus, with 2 distoanterior bunches of setae and numerous posterior marginal setae (fig. 7D). Propodus trapezoid, broader than long (ratio: 95:90), at posterior margin with 14 transverse rows of setae (fig. 7E); palm slightly convex, inclined hardly over half of propodus-length, defined on outer face by corner S-spine accompanied laterally by 3 slender L-spines and 6-7 corner facial M-setae (fig. 7F). Dactylus reaching posterior margin of propodus, along outer margin with row of setae (1-2-1-1-1-1-2-2-1-2-1-1-1-2, or 2-1-1-2-3-3-2-2-2-1-3), and very short setae at inner margin (fig. 7E).

Pereopods 3-4 moderately slender. Pereopod 3 article 2 with proximoanterior and proximoposterior marginal long setae, distoanterior and distoposterior marginal setae are shorter (fig. 6D). Articles 4-6 of different length (ratio: 50:32:39); article 4 with 4 bunches of posterior marginal setae (the longest setae slightly exceeding width of article itself) and 4 groups of short anterior marginal setae; article 5 with 5 groups of short spines and single short setae; article 6 along posterior margin with 5 bunches of short spines or setae. Dactylus much shorter than article 6 (ratio: 17:39), at inner margin with one short spine, at outer margin with one median seta; nail nearly as long as pedestal (fig. 6E).

Pereopod 4 rather similar to pereopod 3 but hardly shorter, and hardly shorter setae (fig. 6F). Articles 4-6 of different length (ratio: 45:32:36); dactylus much shorter than article 6 (ratio: 16:37), at inner margin with one short spine, at outer margin with one median seta; nail nearly as long as pedestal (fig. 6G).

Pereopods 5-7 moderately slender, scarcely spinose. Pereopod 5 remarkably shorter than pereopods 6 and 7; article 2 longer than broad (ratio: 58:33), anterior slightly convex margin with 5-6 groups of short spine-like setae, posterior margin hardly concave in the middle, provided with nearly 14 short setae, ventroposterior lobe absent (fig. 8A). Articles 4-6 of different length (ratio: 38:42:42), at both margins with short spines and setae. Article 2 longer than article 6 (ratio: 58:38). Dactylus much shorter than article 6 (ratio: 13:42), at inner margin with one small spine (fig. 8B), at outer margin with one median seta; nail as long as pedestal.

Pereopod 6: article 2 remarkably longer than broad (ratio: 77:37), anterior slightly convex margin with 8 groups of short setae, posterior margin straight in the middle, bearing nearly 17 short setae, ventroposterior lobe not developed (fig. 8C). Articles 4-6 of different length (ratio: 49:61:68); articles 4 anterior margin with several single or pairs of short setae and distal short spine, posterior margin with 4 groups of short spines; articles 5-6 at both margins with groups of spines and setae much shorter than width of articles themselves. Article 6 slightly shorter than article 2 (ratio: 77:68). Dactylus much shorter than article 6 (ratio: 21:68), with one short spine at inner margin and one median seta at outer margin (fig. 8D); nail shorter than pedestal (ratio: 37:48).

Pereopod 7: article 2 much longer than broad (ratio: 76:39), anterior margin almost straight, with 7 groups of short spine-like setae, posterior almost straight margin with nearly 14 short setae replaced with several short spines (fig. 8E), ventroposterior lobe absent. Articles 4-6 of different length (ratio: 55:57:61) at both margins with groups of short spins and /or short setae (fig. 8E). Article 6 shorter than article 2 (ratio: 76:61), dactylus much shorter than article 6 (ratio: 18:61), with one short spine at inner margin and 2 median plumose setae at outer margin (fig. 8F), nail shorter than pedestal (ratio: 38:47) [my impression is that figured pereopod 7 is with partially regenerated some distal articles].

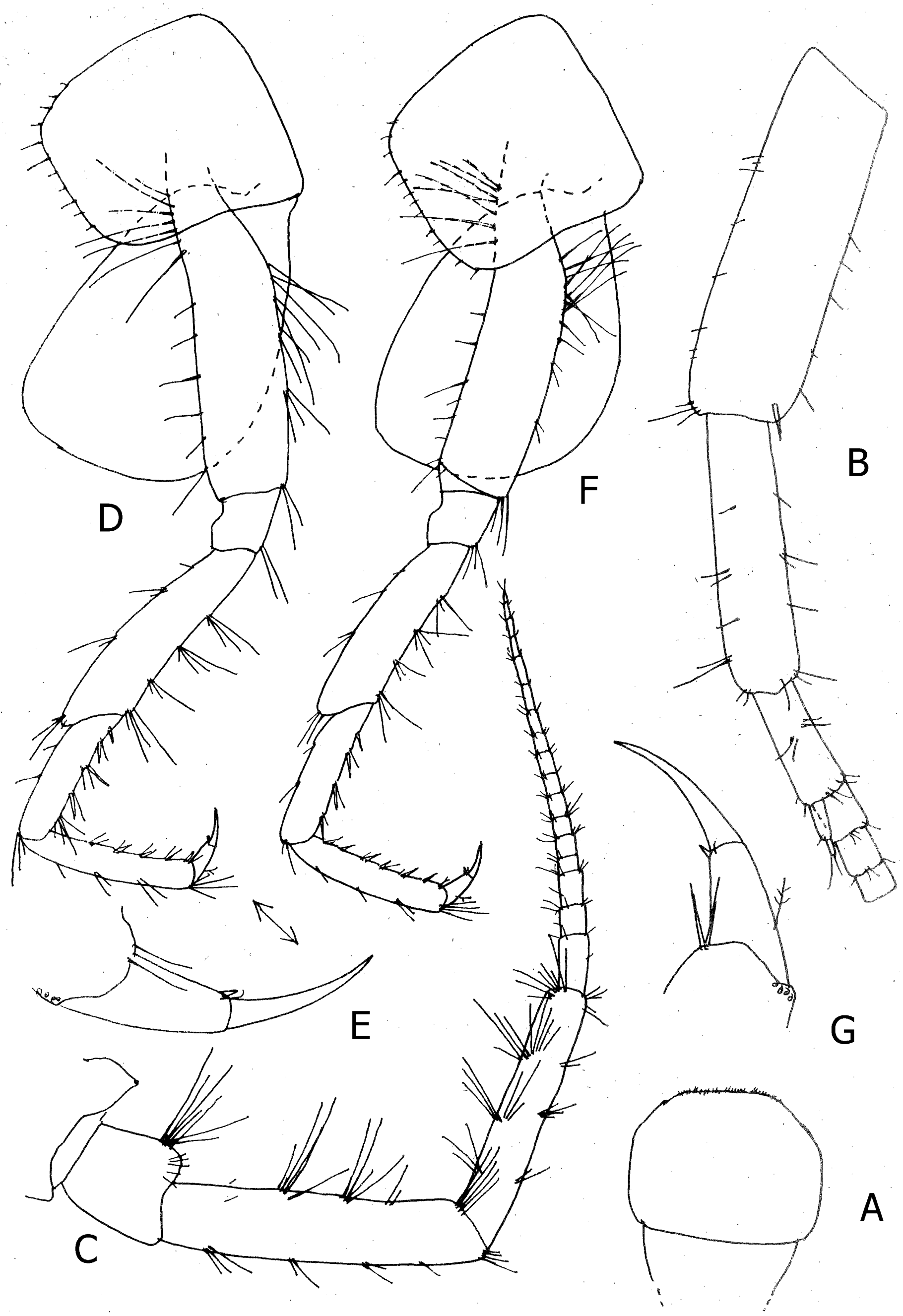

Fig. 6. *Niphargus* (*Niphargus*) *gottscheeanensis* Delić et al. 2017, Źeljnske jame Cave, Kočevje, male 20.0 mm: A= labrum; B= antenna 1; C= antenna 2; D-E= pereopod 3; F-G= pereopod 4.

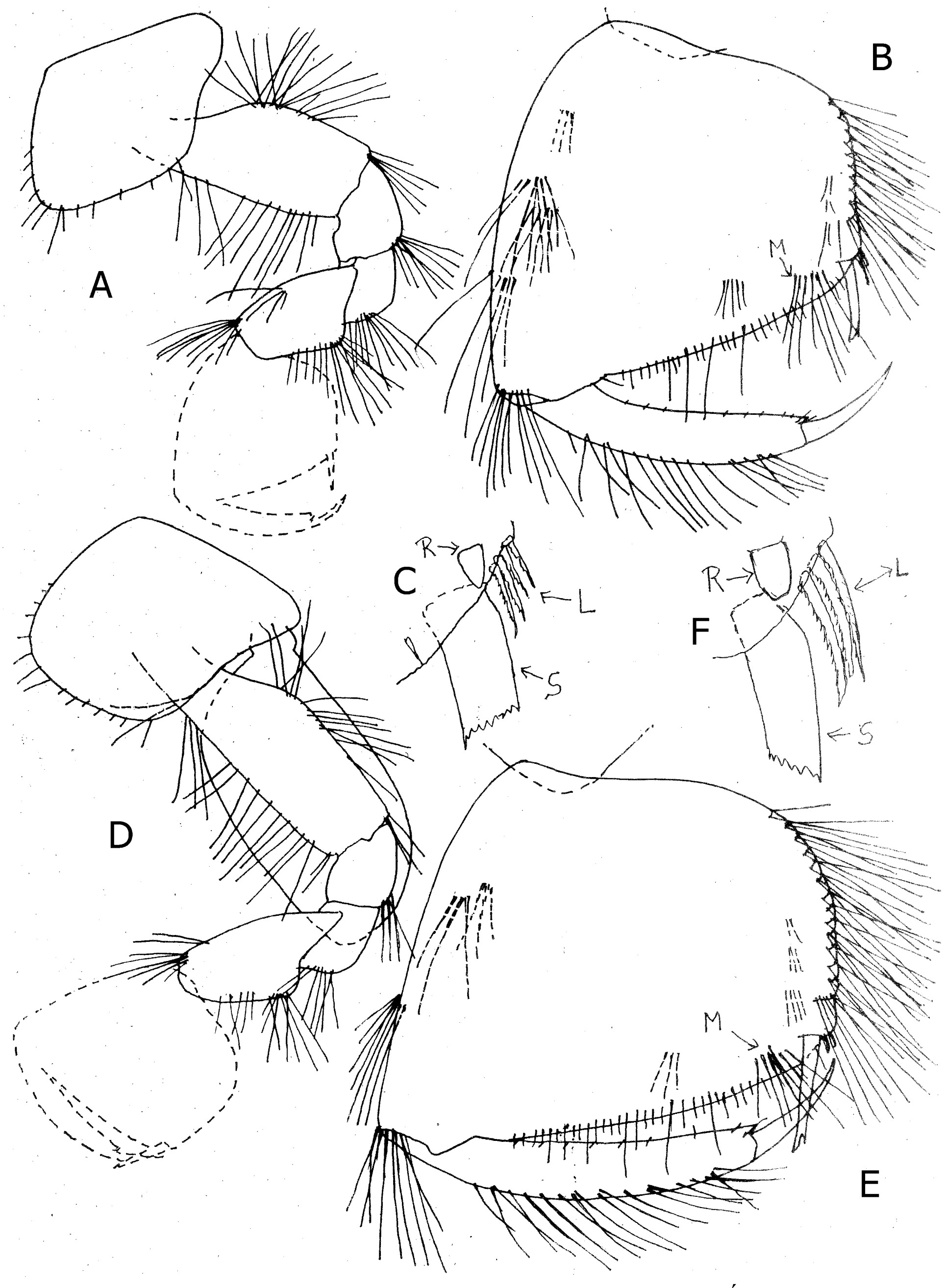

Fig. 7. *Niphargus* (*Niphargus*) *gottscheeanensis* Delić et al. 2017, Źeljnske jame Cave, Kočevje, male 20.0 mm: A-B= gnathopod 1; outer face; C= distal corner of gnathopod 1-propodus, inner face [S= corner S-spine; L= lateral L-spines; M= corner facial M-seta; R= subcorner R-spine, inner face]; D-E= gnathopod 2; outer face; F= distal corner of gnathopod 2-propodus, inner face [S= corner S-spine; L= lateral L-spines; M= corner facial M-seta; R= subcorner R-spine, inner face].

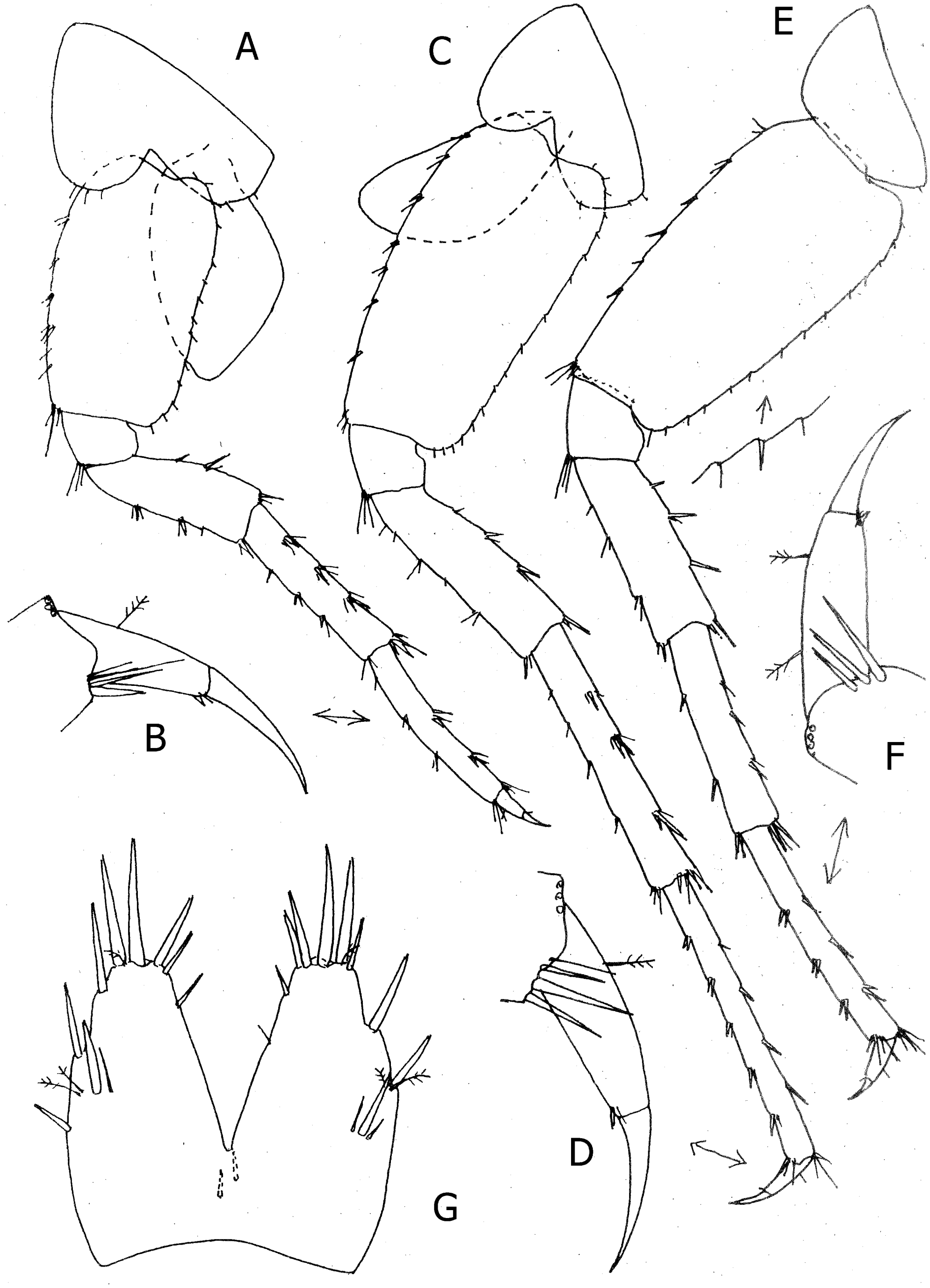

Fig. 8. *Niphargus* (*Niphargus*)"*gottscheeanensis* Delić et al. 2017, Źeljnske jame Cave, Kočevje, male 20.0 mm: A-B= pereopod 5; C-D= pereopod 6; E-F= pereopod 7; G= telson.

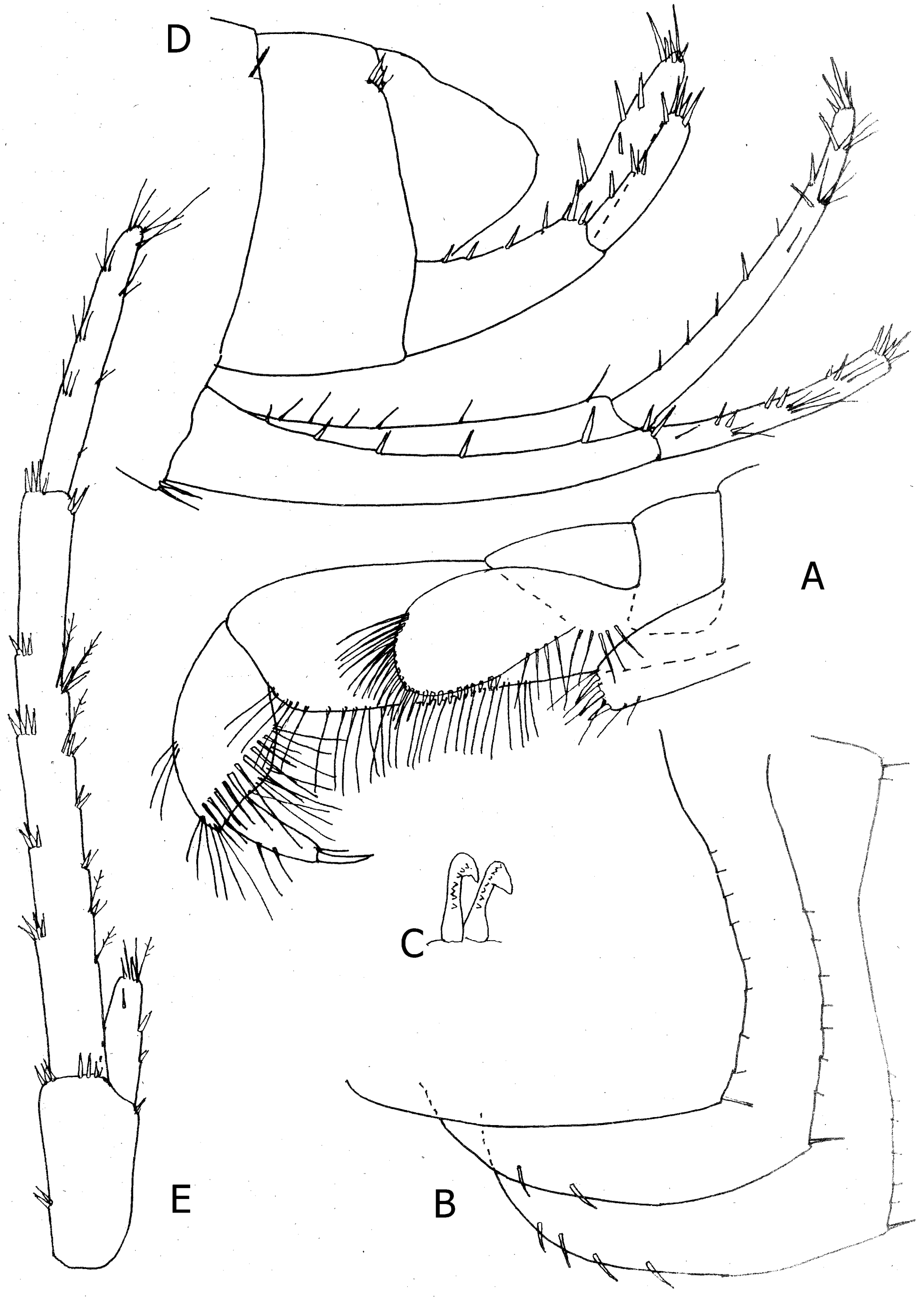

Fig. 9. *Niphargus* (*Niphargus*) *gottscheeanensis* Delić et al. 2017, Željnske jame Cave, Kočevje, male 20.0 mm: A= maxilliped. B= epimeral plates 1-3; C= retinacula; D= urosome with uropods 1-2; E= uropod 3.

Pleopods with 2 retinacula. Peduncle of pleopod 1 with 5 anterior simple setae sitting in the middle of anterior face of peduncle (fig. 9 I). Peduncle of pleopod 2 with 1 distoanterior median simple seta and 4 outer facial plumose setae (fig. 10J). Peduncle of pleopod 3 at posterior margin with 4-5 simple setae and 9-11 outer facial plumose setae (fig. 10K).

Uropod 1: peduncle relatively long, with dorsoexternal row of spines and dorsointernal row of setae; inner ramus elongated but shorter than peduncle, outer (dorsal) margin with row of several slender short spines and distal bunch of 5 short spines, as well as with 2-3 subdistal groups of short simple setae (fig. 9D); outer ramus exceeding half of inner ramus, with several lateral and 5 distal short spines as well as with 3-4 groups of short simple setae.

Uropod 2: peduncle with dorsal row of spines; inner ramus distinctly longer than outer one, both rami with short lateral and 5 distal short spines.(fig. 9D).

Uropod 3 long; peduncle longer than broad; with lateral and distal short spines; inner ramus shorter than peduncle, with single lateral and distal spines; outer ramus long, 2-articulated, first article at outer margin with 5 bunches of short spines (fig. 9E), at inner (mesial) margin with several groups of short spines mixed with single plumose setae; second article shorter than first one (ratio: 62:130), with bunches of short simple setae at margins and tip. Telson slightly broader than long, gaping; each lobe with row of 5 distal and outer and inner marginal spines, as well as with one facial spine accompanied by 1-2 short setae; a pair of short plumose setae appears near the outer middle of each lobe (fig. 8G).

Coxal gills on gnathopod 2 and pereopods 3-4 large (figs. 6D,E; 7D), these of pereopods 5-6 smaller (fig. 8A, C).

FEMALE 18 mm with setose oostegites: Mainly likes the male; metasomal segments 1-3 with 4 dorsoposterior marginal setae each (fig. 11E). Urosomal segment 1 with one seta on each dorsolateral side; urosomal segment 2 with 3 spines and one seta on each dorsolateral side, urosomal segment 3 naked (fig. 12G). Urosomal segment 1 with one group of 3 ventroposterior spine-like setae near basis of peduncle of uropod 1 (fig. 12G).

Epimeral plates 1-2 quadrate, with slightly convex posterior margin bearing nearly 5 short setae and poorly marked ventroposterior corner (fig. 11E). Epimeral plate 3 quadrate, with almost straight inclined posterior margin with nearly 10 short setae and well marked ventroposterior corner; epimeral plate 2 with 3, and epimeral plate 3 with 4 subventral spines. Antenna 1 poorly exceeding half of body, main flagellum with 29 articles; flagellum of antenna 2 with 12 articles.

Mouthparts like these in male. Mandibular palpus article 2 with 15 setae, article 3 with nearly 30 D-setae, 10 A setae, 10 B setae (2-3-3-1-1) and 7 E setae.

Maxilla 1 inner plate with 5 setae, outer plate with 7 spines [6 spins with one tooth, 1 spine with 3 teeth), palpus short, with 11 setae. Maxilliped like that in male.

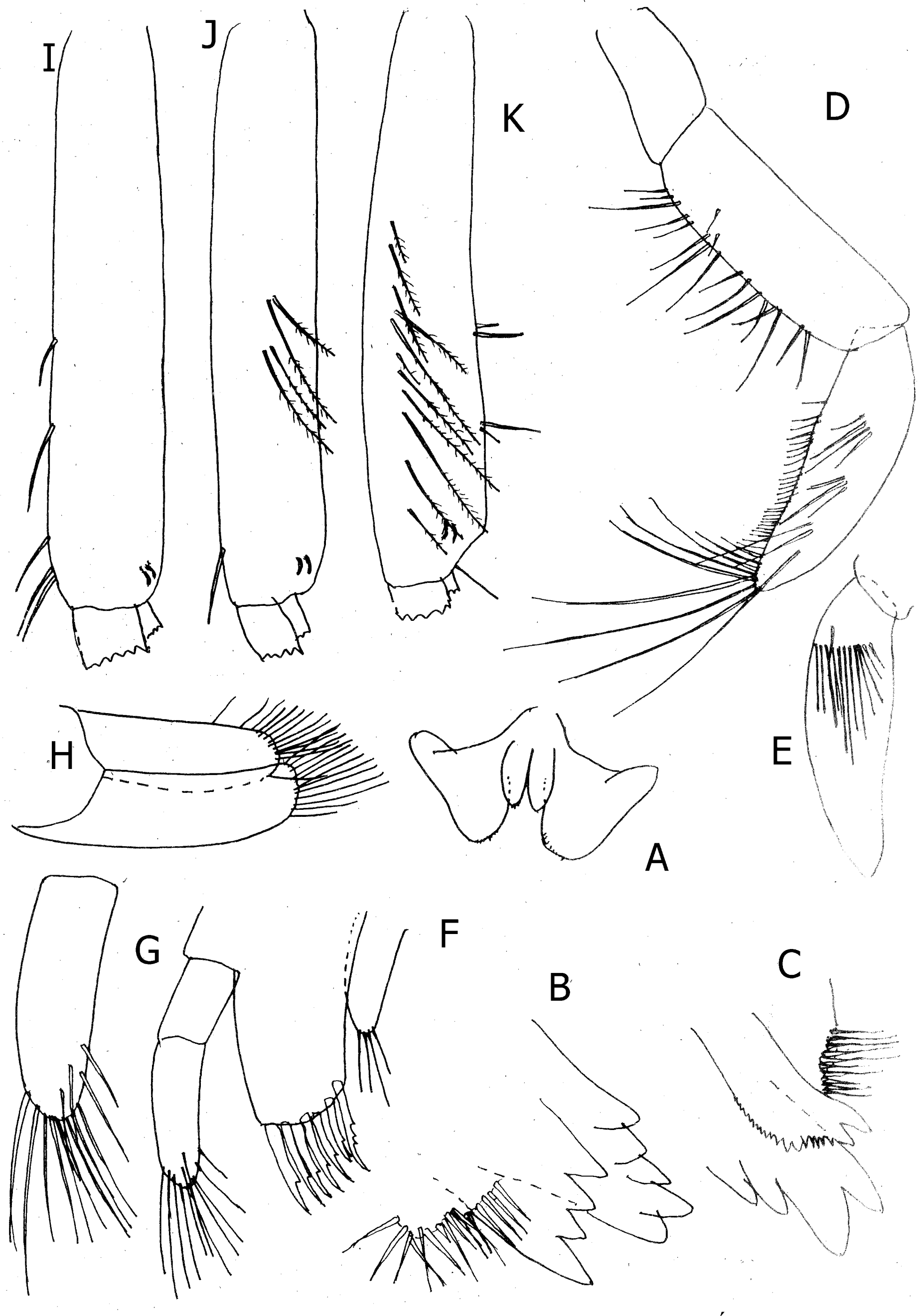

Fig. 10. *Niphargus* (Niphargus) *gottscheeanensis* Delić et al. 2017, Źeljnske jame Cave, Kočevje, male 20.0 mm: A= labium; B= left incisor with lacinia mobilis and rakers; C= right incisor with lacinia mobilis and rakers; D= mandibular palpus inner face; E= distal article of mandibular palpus, outer face; F-G= maxilla 1; H= maxilla 2; I= pleopod 1-peduncle; J= pleopod 2-peduncle; K= pleopod 3-peduncle.

Coxae 1-4 are slightly longer than these in males. Coxa 1 hardly longer than broad (ratio: 37:35), with narrowly subrounded ventroanterior corner and provided with nearly 17 short setae (fig. 11A); coxa 2 slightly longer than broad (ratio: 47:40), with nearly 15 marginal setae (fig. 11B); coxa 3 longer than broad (ratio: 52:40), with nearly 12 marginal setae (fig. 11C); coxa 4 longer than broad (ratio: 50:45), with nearly 11 marginal setae, ventroposterior lobe absent (fig. 11D).

Coxae 5-7 like these in male. Coxa 7 with one posterior marginal seta (fig. 12A). Gnathopod 1 articles 2-5 like these in male. Propodus trapezoid, slightly longer than broad (ratio: 80:72), at posterior margin with 8 transverse rows of setae, at anterior margin with 2 lateral and one distal bunch of setae (fig. 11F); palm slightly convex, inclined rather over half of propodus-length, defined on outer face by corner S-spine accompanied laterally by 4 L-spines and 7 corner facial M-setae; on inner face by one subcorner R-spine (fig. 11G). Dactylus reaching posterior margin of propodus, at outer margin with row of single or paired setae (1-2-1-2-1-1-2-1-1-1-2), at inner margin with row of very short setae (fig. 11F).

Gnathopod 2: articles 2-5 like these in male. Propodus trapezoid, less inclined than that in male, rather broader than long (ratio: 95:82), along posterior margin with 12 transverse rows of setae, at anterior margin with 2 lateral and one distal bunch of setae (fig. 11H). Palm slightly convex, inclined nearly half of propodus-length, defined on outer face by corner S-spine accompanied laterally by 3 slender L-spines and corner facial 6 M-setae, on inner face by one subcorner R-spine. Dactylus reaching posterior margin of propodus, at outer margin with row of setae (1-1-3-2-2-3-2-2-1-1-2), at inner margin with row of very short setae (fig. 11H).

Pereopods 3-4 like these in male. Pereopod 3 article 2 with numerous long anterior and posterior marginal setae (the longest setae exceeding diameter of article itself); article 3 with distoposterior bunch of setae exceeding length of article 3. Articles 4-6 of different length (ratio: 50:30:40), with pilosity like that in these of male. Dactylus much shorter than propodus (ratio: 22:42), with small spine at inner margin and one median plumose seta at outer margin, nail almost as long as pedestal.

Pereopod 4 like pereopod 3 with scarcely shorter various setae.

Pereopods 5-6 like these in male. Pereopod 7 as long as pereopod 6, with article 2 longer than broad (ratio: 88:43), anterior poorly convex margin with row of short spines mixed with single short setae, posterior slightly convex margin with nearly 15 short setae (some setae are replaced with short spines (fig. 12A), ventroposterior lobe absent. Articles 4-6 of different length (ratio: 43:65:80), along both margins with several bunches of short spines mixed often with single short setae (fig. 12B). Article 2 is longer than article 6 (ratio: 88:80). Dactylus much shorter than article 5 (ratio: 25:80), at inner margin with one weak spine near basis of the nail, at outer margin with 2 single plumose setae (fig. 12C), nail slightly shorter than pedestal (ratio: 33:45).

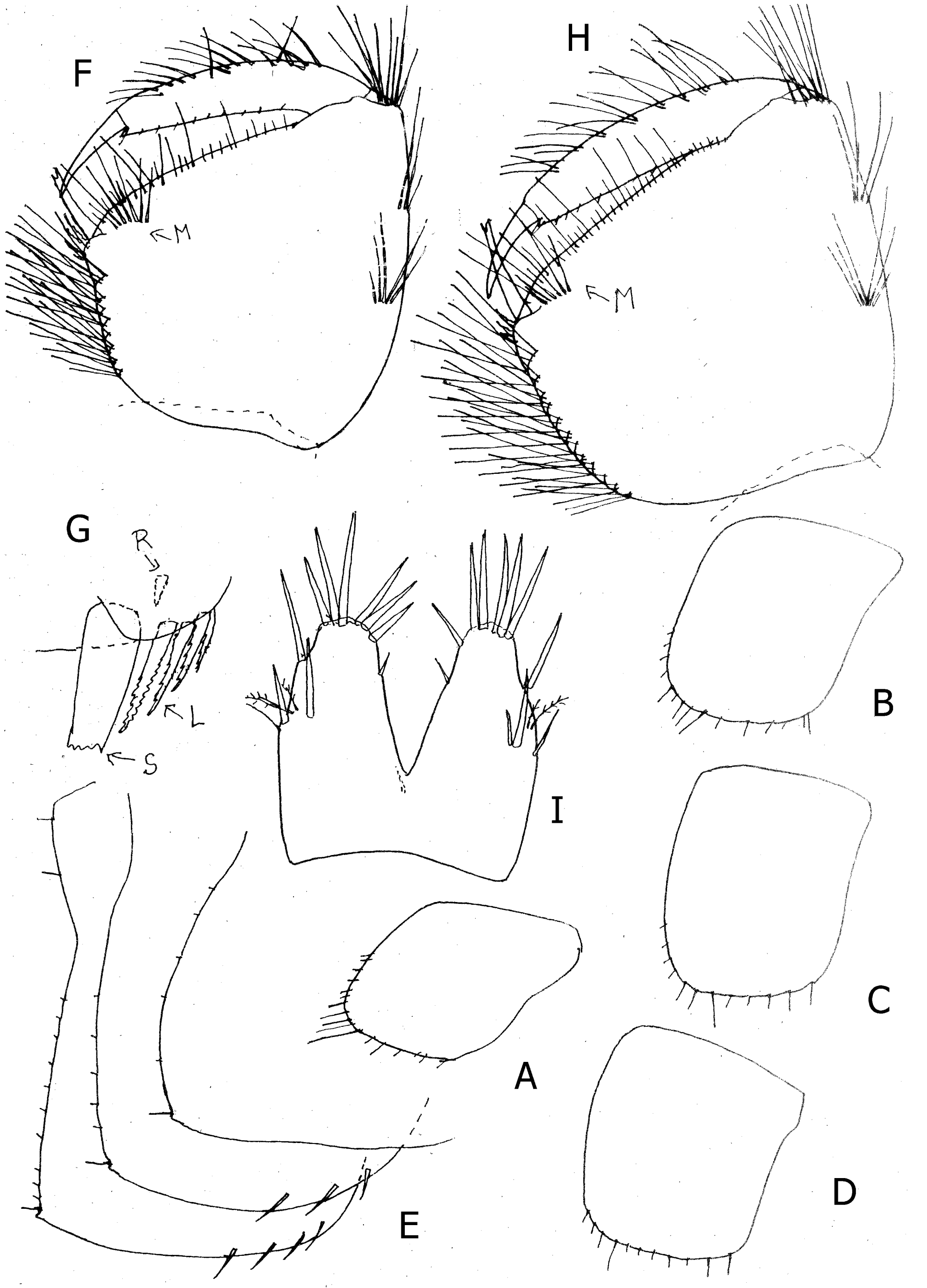

Fig. 11. *Niphargus* (*Niphargus*) *gottscheeanensis* Delić et al. 2017, Źeljnske jame Cave, Kočevje, female 18.0 mm: A= coxa 1; B= coxa 2; C= coxa 3; D= coxa 4; E= epimeral plates 1-3; F= gnathopod 1-propodus, outer face; G= distal corner of gnathopod 1-propodus, outer face [S= corner S-spine; L= lateral L-spines; R= subcorner R-spine, inner face].

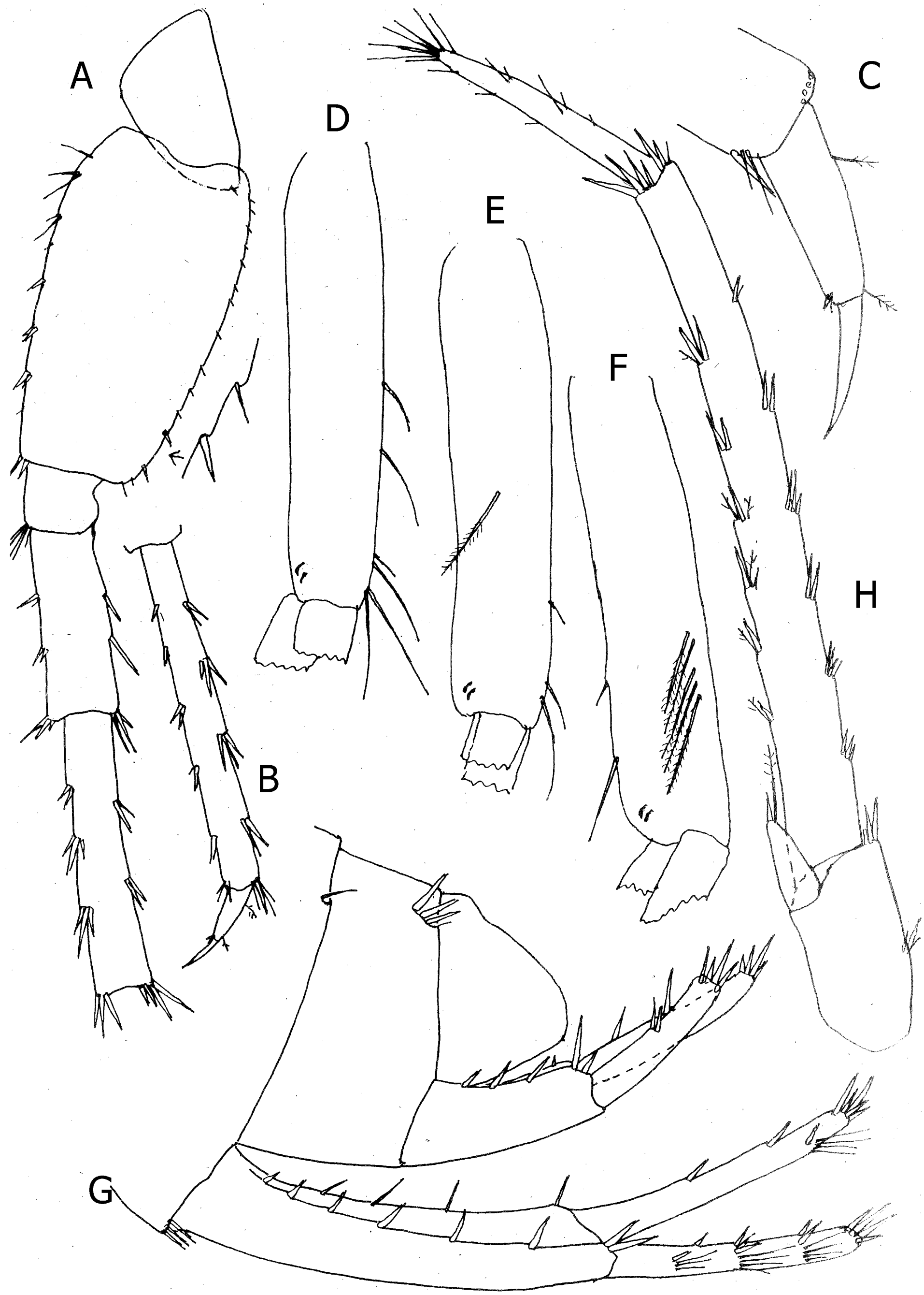

Fig. 12. *Niphargus* (*Niphargus*) *gottscheeanensis* Delić et al. 2017, Źeljnske jame Cave, Kočevje, female 18.0 mm: A-B-C== pereopod 7; D= pleopod 1-peduncle; E= pleopod 2-peduncle; F= pleopod 3-peduncle; G= urosome with uropods 1-2; H= uropod 3.

Pleopods 1-3 with 2 retinacula. Peduncle of pleopod 1 with 6 simple setae in the middle of anterior face of peduncle (fig. 12D); peduncle of pleopod 2 with 3 anterior simple setae and one outer facial plumose seta (fig. 12E). Peduncle of pleopod 3 with 2 4 posterior marginal simple setae and with 5-6 outer facial plumose setae.(fig. 12F).

Uropod 1: peduncle with dorsoexternal row of spines and dorsointernal row of setae (fig. 12G); inner ramus shorter than peduncle, with single lateral and 5 distal short spines, as well as with one bunch of short subdistal simple setae; outer ramus only slightly shorter than inner one, with 3 lateral spines and distal bunch of 4 spines, as well as with 3 lateral bunches of simple setae

Uropod 2: inner ramus slightly longer than outer one, both rami with lateral and distal short spines (fig. 12G).

Uropod 3: peduncle longer than broad, with one lateral and 2-3 distal spines; inner ramus scale-like, much shorter than peduncle with distal spine and plumose seta; outer ramus 2-articulated, elongated, first article along inner and outer margin with row of nearly 7 groups of short spines, at inner margin mixed with single very short plumose setae (fig. 12H); second article shorter than first one (ratio: 57:160), with short lateral single setae and distal bunch of rather longer simple setae.

Telson slightly broader than long, gaping; incised almost 2/3 of telson-length; each lobe with 5-6 distal and 1-2 outer and inner marginal spines, as well as 1-2 facial spines; a pair of short plumose setae attached medially at outer lateral margin of each lobe (fig. 11 I).

Coxal gills like these in male. Oostegites broad, occur on mesosomal segments 2-5.

VARIABILITY.

Number of outer marginal setae on dactylus in gnathopods 1-2 rather variable; inner plate of maxilla 1 with 4-5 setae, palpus with 11-14 setae. Dactylus of pereopod 7 with 1-2 median setae at outer margin; article 6 of pereopod 7 usually as long as that in pereopod 6, seldom shorter (regeneration?). Urosomal segment 1 at ventroposterior corner usually with one spine, seldom replaced with 2-3 spine-like setae. Telson always with distal, lateral and facial spines. The number of plumose setae on peduncle of pleopods is rather variable but present.

KOMPOLJSKA jama Cave (male 15 mm, female 20 mm: mainly agree with specimens of Źeljnske jame Cave, but also with these of Podpeška jama Cave: narrowed article 2 of pereopods 5-7 with posterior marginal setae sometimes replaced by single small spine; peduncle of pleopod 1 with 3-5 anterior setae, that of pleopod 2 with 1-4 anterior seta; peduncle of pleopod 3 with 3 posterior simple setae and 3-5 facial plumose setae.

Maxilla 1 inner plate 3-4 setae, palpus short, with 11-13 setae, gnathopods 1-2 propodus with one S-spine, 3 slender L- spines, 5-6 facial M-setae and one subcorner R-spine. In males uropod 1 outer ramus reaching 2/3 of inner one (male 15 mm); uropod 2 inner ramus only slightly longer than outer one; one ventroposterior spine on urosomal segment 1 near basis of uropod 1-peduncle.

In females uropod 1 outer ramus reaching 5/6 of inner ramus, uropod 2 outer ramus reaching 4/5 of inner one. Uropod 3 in male of 15 mm outer ramus second article reaching 3/5 of first one, in females reaching 1/2 to 1/4 of first article.

Epimeral plate 3 angular or hardly acute; epimeral plates 1-2 with less convex posterior margin. Dactylus of pereopods 5-7 strong, with one slender spine at inner margin. Telson lobes with one facial spine, 3-4 distal and 0-2 inner and/or outer marginal spines.

LOCALITIES CITED : SLOVENIA:

Delić et al. 2017: Podpeška jama Cave, Podpeč, Dobrepolje; Spring SW from Sušje, Ribnica; Cave in quarry, Vinica, Črnomelj; Židovske kuće, Budinjak, Žumberak; Željnske jame Cave, Željne, Kočevje; springs NW of Kočevska reka River; Kočevska reka near Kočevje; spring near Obvrh, Mirtoviči, Osilnica;

?Kompoljska jama Cave, Slovenia (new).

REMAKS AND AFFINITIES.

The splitting *N. podpecanus* into three different species, (but without analyze of its morphological characters), the absence of different known morphological characters between *N. podpecanus* and *N. gottscheeanensis* and finding both taxa in the same locality (Podpeška jama Cave; Źeljnske jame Cave; spring near Obrh); request further studies and confirmation of its taxonomical status.

REFERENCES

Balazuc, J. 1957. Notes sur les Amphipodes souterraines. - Notes Biospel. I. 12: 67-80, Paris (1958), figs. 1-2. I. Un nouveau Gammaride troglobie de l Ariège. *Salentinella gineti* n. sp. II. Additions à la faune gallo-rhénane.- Notes Biospel. 12/2: 67-80.

Barnard, J.L & Barnard, C.M. 1983. Freshwater amphipods of the World. I. Evolutionary patterns. II. Handbook and bibliography.- Hayfield Associates: Mt. Vernon, Virginia, 1983, pp. XIX +849 pages, 50 figs., 7 graphs, 98 maps, 12 tables.

Bréhier, F. & Jaume D. 2009. A new species of *Pseudoniphargus* (Crustacea, Amphipoda, Melitidae) from an anchialine cave on the French Mediterranean coast. - Zoosystema 31 (1): 17-32.

Delić, T., Trontelj, P., Rendoš, M. & Fišer, C. 2017. The importance of naming cryptic species and the conservation of endemic subterranean amphipods.- Scientific Reports 7 (1): 1-12.

Ferreira, D., Malard, F., Dole-Olivier, M.J. & Gibert, J. 2007. Obligate groundwater fauna of France: diversity patterns and conservation implications.-Biodiversity and Conservation, 16: 567–596 Springer 2007 DOI 10.1007/s10531-005-0305-7.

Ginet, R. 1995/96. Bilan systematique du genre *Niphargus* en France. Especes repertories jusqu`en 1990 dans les eaux souterraines de la France.- Universite Claude-Bernard Lyon-I. Pp. 1-238 (realisation: Florian Malard, 1991, complete 1995). Soc. Linneenne Lyon.

Karaman, G. 1969. XXVII. Beitrag zur Kenntnis der Amphipoden. Arten der Genera *Echinogammarus* Stebb. und *Chaetogammarus* Mart. an der jugoslawischer Adriaküste. - Glasnik Republičkog zavoda za zaštitu prirode i Prirodnjačke zbirke u Titogradu, 2: 59-84, 51 figs.

Karaman, G. 1972. Le probleme du Genre *Niphargus* en Yougoslavie. - Actes du I-er Colloque International sur le genre *Niphargus*-Verona, 15-19 Aprile 1969, Museo Civico di Storia Naturale, Verona, Memorie fuori serie, 5: 1-10.

Karaman, G. 1974. Catalogus Faunae Jugoslaviae, Crustacea Amphipoda (Contribution to the Knowledge of the Amphipoda 60).- Consilium Academiarum Scientiarum Rei Publicae Socialisticae Foederativae Jugoslaviae, Academia Scientiarum et Artium Slovenica, Ljubljana, 3 (3): 1-44.

Karaman, G. 1977. Contribution to the Knowledge of the Amphipoda 90. Revision of *Gammarus balcanicus* Schäf. 1922 in Yugoslavia (Fam. Gammaridae). - Poljoprivreda i šumarstvo, Titograd, 23 (4): 37-60, figs. I-VII.

Karaman, G. & Ruffo, S. 1986. Amphipoda: *Niphargus*-Group (Niphargidae sensu Bousfield, 1982), in: Botosaneanu, L. (edit.): Stygofauna Mundi, A Faunistic, Distributional, and Ecological Synthesis of the World Fauna inhabiting Subterranean Warers (including the Marine Interstitial), Leiden, E. J. Brill/ Dr. W. Backhuys, pp. 514-534.

Karaman, G. 2012. Further investigations of the subterranean genus *Niphargus* Schiödte, 1849 (fam. Niphargidae) in Serbia. (Contribution to the Knowledge of the Amphipoda 264). - Agriculture and Forestry, Podgorica, 58 (2): 45-64, 7 figs.

Karaman, S. 1952. Podrod *Stygoniphargus* u Sloveniji i Hrvatskoj. [Das Subgenus *Stygoniphargus* in Slovenien und Kroatien]. - Prirodoslovna istraživanja, Odjel za prirodne i medicinske nauke, Jugoslavenska Akademija znanosti i umjetnosti Zagreb, 25: 3-38, figs. 1-62.

Premate, E., Borko, Š., Delić, T., Malard, F., Simon, L. & Fišer, C. 2021. Cave amphipods reveal co-variation between morphology and trophic niche in a low-productivity environment.- Freshwater Biology, 00: 1–13.

Ruffo, S. 1954. Anfipodi di acque interstiziali raccolti dal Dr C. Delamare Deboutteville in Francia, Spagna e Algeria. (Studi sui crostacei anfipodi XXXIX). - Vie et Milieu, 4 (4): 669-681.

Nedeljković, J., Poduška, Z., Krgović, D., Nonić, D. (2022): Critical success factors of small and medium enterprises in forestry and wood industry in the Južnokučajsko forest region. Agriculture and Forestry, 68 (1): 71-93. doi:10.17707/AgricultForest.68.1.04

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CRITICAL SUCCESS FACTORS OF SMALL AND MEDIUM ENTERPRISES IN FORESTRY AND WOOD INDUSTRY IN THE JUŽNOKUČAJSKO FOREST REGION

SUMMARY

Contractors for forest services play an important role in the forestry sector and, nowadays, are a common model of performing activities in forests. In addition, they represent a link between forest owners and enterprises within the wood industry. This research aims to study the characteristics of small and medium enterprises (SMEs) in the forestry and wood industry and the organization of their business processes in the Južnokučajsko forest region (JKFR), as internal critical success factors. JKFR was chosen because it has a high forest cover, a large share of privately owned forests, and the largest primary wood processing company in East Serbia is located within the region and developed furniture industry. Data were collected by conducting surveys with 43 representatives of SMEs from the forestry, primary and final wood processing sectors, which operate on the territory of JKFR. The following internal critical success factors are analysed: characteristics of the enterprises and organization of business processes. The results show that most of the forestry and wood industry enterprises were established after 2000. All enterprises from the forestry and primary wood processing sector belong to the category of micro-enterprises, while 14% of final wood processing enterprises belong to the category of small, and the rest to micro-enterprises. The largest number of SMEs in forestry (77.8%) perform activities in state forests, and 66.7% in private forests. Around ½ SMEs in forestry perform work without long-term contracts with other enterprises. However, most (88.9%) of these enterprises perform forest work independently (using their own machinery). Primary wood processing enterprises most often procure raw materials from private forest owners (77.8%), and final

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wood processing enterprises from sawmills (78.6%). Most wood industry enterprises have their own processing facilities, while others perform processing in cooperation with other enterprises. The most frequent buyers of products from primary processing enterprises are individuals, while most (78.6%) of final wood processing enterprises sell products through retail.

Keywords: small and medium enterprises, forestry, wood industry, business processes, critical success factors, Južnokučajsko forest region

INTRODUCTION

The entire business of an organizational system (e.g. enterprise) is done through business processes (Janićijević et al., 2019). They represent "…*a set of related activities and decisions, which are carried out to achieve a measurable goal of the organization, last a certain time and consume some input resources, turning them into specific products or services relevant to the customer or user*" (Brumec, 2011). Basic operational business processes (procurement, processing / production, selling) occur in manufacturing enterprises. Procurement is an operational business process procuring raw materials for further processing and/or selling. Production/processing is an operational business process within which the raw material is processed into a final or semi-finished product or simply packaged and sold on domestic and international markets. Selling is a business process which includes the sale and delivery of products to customers. This includes all business activities that organizations carry out to sell goods or services (Nonić, 2015, Nedeljković, 2021). Service companies, "…*which include specialized service companies in forestry, have a specifically organized business, in which such business processes cannot be clearly distinguished*" (Nedeljković, 2021).

SMEs in the forest sector have their own specifics. Some specifics are partly opposed to the theory of creating new forms of enterprise. While other specifics are recognized as imitations of natural ecosystems, which Moore (1988) called "business ecosystem". The theoretical aspects of creating new forms of enterprise assume that managers calibrate the mindset to "more and more", not caring about the resource (Moore, 1998). It is in contrast to forestry where moderation and sustainability of resources is the leading concept. Another aspect is the mimicry of new forms of enterprise in a form of imitation of natural ecosystems. These business ecosystems are also called economic communes aims to unite "… *customers, suppliers, lead producers, and other stakeholders - interacting with one another to produce goods and services*" (Moore, 1998). These theoretical aproaches led to stattement that small and medium enterprises (SMEs) must create their own bussines ecosystem to attract suppliers, partners and financial capital to cooperative networks (Moore, 1993). All of abovemention approaches lead SMEs to successful business.

Successful businesses are considered those that evolve rapidly and effectively. They must be able to attract resources of all sorts, drawing in capital, partners, suppliers, and customers to create cooperative networks. For a better understanding of the work of a successful company, it has to be viewed not as a member of a single industry but as part of a business ecosystem that crosses a variety of industries (Moore, 1993). Business ecosystems are composed of different subjects (e.g. customers, suppliers, lead producers and other stakeholders) interacting with each another to produce goods and services (Moore, 1998). Business ecosystem "…*gradually moves from a random collection of elements to a more structured community*" (Moore, 1993) where SMEs have an important role working as an external associate of the large companies (Simić et al., 2020).

In forestry, large companies usually hire external associates (small and medium service enterprises) to perform silvicultural and harvesting works, transport raw material, construction and maintenance of forest roads, etc. In this way, production costs are reduced, and it is possible to perform better work due to the specialization of SMEs (Eriksson et al., 2015). Nowadays, contractors for forestry services are a common model of performing works in forestry (Nedeljković, 2021). In some countries (e.g. Finland), these companies have a long tradition. On the other hand, in the countries of Eastern and Southeastern Europe, contractors for forestry services are a relatively new entity in the forestry sector, whose development is "…*closely related to mechanization of logging and transport of raw material in era of transition to a market economy*" (Šporčić & Martinić, 2004). Previous research indicates that in countries across Europe, such as Sweden, Finland, Germany and Slovakia, contractors for forestry services are most often small businesses, or, in most cases, micro-enterprises that rarely have more than one employee (Štěrbova & Kovalčik, 2020).

In Serbia, public enterprise (PE) "Srbijašume" performed forest harvesting activities, until 1995, mostly by own workforce, In the period 1996-2002, production of wood assortments by own workforce participated with 21.7% to 30.0% in the total amount of produced wood assortments. After that, the share of production of wood assortments by own workforce decreased, and in 2020 it amounted to only 3.9% of the total amount of produced wood assortments. On the other hand, in 2020, service companies participated with 72.8%, and standing timber sale (self-processing) with 23.2% of the total amount of produced assortments (Aleksić, 2021).

The process of restructuring the PE "Srbijašume", which began in 2001, aimed, among other things, at reducing labour costs (due to the excessive number of employees) and costs associated with the purchase and maintenance of its own machinery. In that period, workers were offered to "…*take over part of the mechanization, and, eventually, become business partners of the PE in the harvesting and similar activities*", which "…*led to the formation of a number of service oriented SMEs*" (Nonić, 2015). According to the available data, the PE "Srbijašume", through a tender, contracts about 500 service enterprises, which perform cutting, production, extraction and transportation of wood assortments, as well as more than 40 companies, which perform silvicultural works (PE "Srbijašume", 2021).

Contractors for forestry services have an impact on ensuring a better living standard for Serbian citizens, especially those from rural areas (Ranković et al., 2012). In addition, there are more than 3,700 wood processing enterprises in Serbia, with more than 52,000 employees. Of the total number of these enterprises, 49% are sawmills, which participate with almost 20% in the total exports of the wood industry sector. It should be noted that "…*more than 90% of enterprises in the wood processing and furniture industry are privately owned, and they are located mainly in the central parts of Serbia*". The wood industry sector participates with 1.4% in the gross domestic product of Serbia, while the share of the wood industry in total exports is about 7.5%, "…*with a positive growth trend in recent years*" and amounts to €1.2 billion (DAS, 2021).

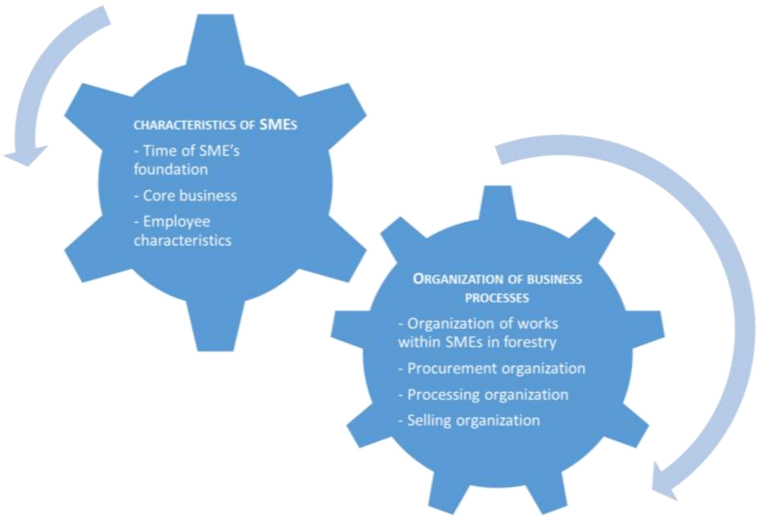
FACTORS AFFECTING SMEs BUSINESS

Critical success factors are defined as "…*those characteristics, conditions, or variables that when properly sustained, maintained, or managed can have a significant impact on the success of a firm competing in a particular industry*" (Leidecker, Bruno, 1984), i.e. "… *those key areas that ensure successful competitive performance for the organization*" (Duarte Alonso & Kok, 2021).

Previous research has point out that "…*only some specific factors are critical to the success of SMEs and should be the "focal point" of the SMEs efforts as long as they are consistent with their nature as small economic entities competing with big companies in small markets*" (Alfoqahaa, 2018). Also, critical success factors are defined by the type of industry, the business environment in which the small and medium enterprise (SME) operates and customer requirements (Simpson et al., 2012). In the literature, critical success factors are most often grouped into two parts: external (environmental factors) and internal (company factors) (Simpson et al., 2012, Alfoqahaa, 2018, Prasad et al., 2020, Duarte Alonso & Kok, 2021). External factors include: institutional framework (Government), market and society requirements, customer requirements, access to financial and technological resources and information, business environment, etc. Internal factors include: company characteristics, technological equipment and investments in technologies and resources, organizational structure, organizational culture, commitment and support of top management, number of employees and their training (education), business processes, business plan, products and services, cooperation, etc. (Chittithaworn et al., 2011; Alfoqahaa, 2018; Prasad et al., 2020).

In the forestry sector, critical success factors for SMEs were defined by Sanchez Badini et al. (2018). According to these authors, the business of SMEs in forestry is influenced by two groups of factors, external and internal. External factors are: macroeconomic environment, regulatory frameworks, forest law enforcement, tenure security and clarity, devolved management and land use planning rights, markets, natural capital. Internal factors are: financial capital, forest management capacities, business management capacities, organizational capacities, clustering (Sanchez Badini et al., 2018).

For the purposes of this paper, an analytical framework (Scheme 1) is defined. This framework identifies the elements needed for the analysis of internal critical success factors, which include the characteristics of SMEs and the organization of business processes in SMEs in forestry and wood industry.



Scheme 1. Analytical framework

The analytical framework combines the characteristics of SMEs and the organization of business processes in SMEs in forestry and wood industry. Within the characteristics of SMEs, the following factors were observed:

-time of SME's foundation;

-core business;

-employee characteristics

Special attention is paid to the time of SME's foundation as an indicator of the decentralization process of PE "Srbijašume", as well as the core business of the enterprise, because it is assumed that most SMEs belong to service companies.

The following factors were considered within the organization of business processes:

-organization of the works within SMEs in forestry;

-procurement organization;

-processing organization;

-selling organization.

With the analytical framework created in this way, the paper aims to study the characteristics of SMEs in the forestry and wood industry and the organization of their business processes in the Južnokučajsko forest region (JKFR), as internal critical success factors. The purpose of the research is to define the basis for further study of the possibilities of improving the internal critical success factors of SMEs in the forestry and wood industry in the selected forest region (FR). The subject of the research is the attitudes of SME representatives in the forestry and wood industry, registered in the municipalities located in the territory of JKFR, towards the characteristics of SMEs and organization of their business processes.

MATERIAL AND METHODS

A study was conducted at the JKFR (Map 1). This FR was chosen to conduct research for several reasons. The forest cover of JKFR is 39.9%, which is above the national average[2]. The total forest area of this FR is 114,257 ha, with the share of privately owned forests being 64.3% (PE "Srbijašume", 2012). In addition, within the borders of JKFR, there is one of the largest enterprises for primary wood processing in Serbia[3], as well as one of the largest factories in the furniture industry[4]. Due to all mentioned reasons, studying SMEs in this FR could provide the best overview of the current situation in this part of the forestry sector.

Data collection was conducted using face to face survey in the period December 2019 - February 2020, while the entry and processing of data was performed in the period March-April 2020.

Respondents were representatives of SMEs registered in the municipalities[5] within the JKFR. They were selected using census, which was then complemented by snowball sampling (Malhotra, 2020).

The preliminary list of enterprises[6] was determined on the basis of information available in online databases[7]. All representatives of these companies were contacted and with some of them, it was agreed to conduct a survey. Upon completion of the survey, respondents were asked to provide recommendations and contacts of representatives of other enterprises with which they cooperate and engaged in the same or similar activities.

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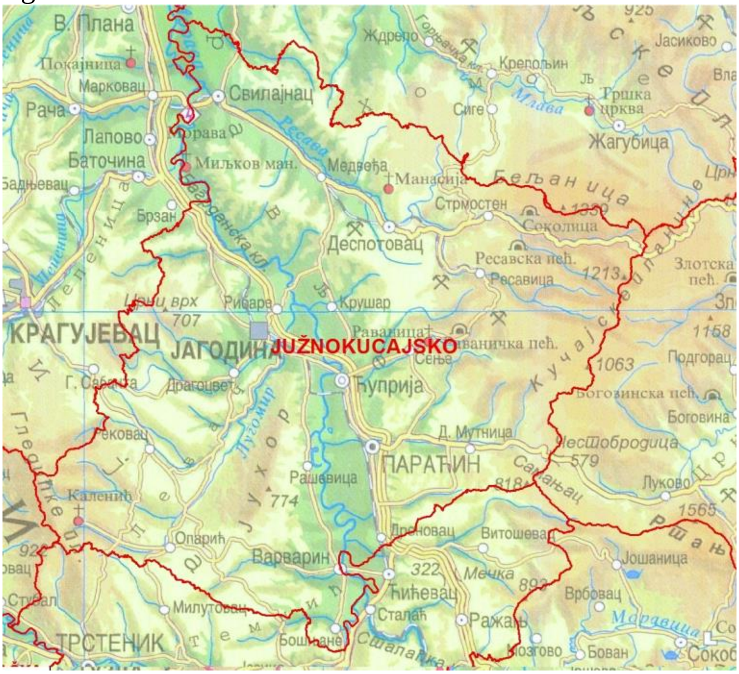
[2] According to official data, forest cover in Serbia is 29.1% (Banković et al., 2009).
[3] "Kronospan SRB d.o.o.", located in munincipality Lapovo (Central Serbia) produces and distributes wood-based panels in more than 40 production facilities around the world (Kronospan, 2021).
[4] "Jela Jagodina d.o.o." is a furniture factory based in Jagodina, founded in 1991. The factory employs over 300 workers (Jela, 2021).
[5] JKFR includes the territory of the City of Jagodina and the municipalities of Despotovac, Paraćin, Ćuprija, Rekovac and Varvarin. Representatives of enterprises from the municipalities of Rekovac and Varvarin did not express interest in participating in the research.
[6] Enterprises which belong to following codes according to business activity: 02.10, 02.20, 02.40, 02.30, 16.10, 16,21, 16.22, 16.23, 31.01, 31.02, 31.09.
[7] A database was used for each activity code by selected city and municipalities from the website https://www.companywall.rs/. After searching and collecting contacts, a single database was formed within the cross-calculation program, which united enterprises with same code of the business activity..

All surveys were conducted in person, using the face-to-face survey method. An overview of enterprise representatives who participated in the research is given in Table 1.



Map 1. Overview of the borders of JKFR (adapted according to the internal documentation of PE "Srbijašume")

Out of the total number of respondents (43), 19 are representatives of SMEs in forestry, 10 SMEs in primary wood processing and 14 SMEs in final wood processing. Two out of 10 primary wood processing enterprises also perform works in forests. Also, three enterprises in final wood processing, perform activities related to primary processing.

The questionnaire consisted of six parts:

1. socio-demographic characteristics of the respondents (questions I-1 to I-8);
2. enterprise's characteristics (questions II-1 to II-5);
3. organization of business processes (questions III-1 to III-15);
4. cooperation and business networking (questions IV-1 to IV-6);
5. frameworks that provide business conditions and support measures (questions V-1 to V-3);
6. conditions in which business activities take place (question VI-1).

For the purposes of this paper, in accordance with the analytical framework, data related to the characteristics of the enterprise and the organization of business processes are presented and analysed (groups II and III).

Table 1. Respondents according to territory and busses activity

City/municipality	business activity code	number of respondents
Jagodina	16.23	1
	02.40	1
	31.01	1
Paraćin	16.23	1
	02.40	3
Ćuprija	16.10	1
	16.23	1
	31.01	1
Svilajnac	16.10	1
	31.01	1
Despotovac	16.10	4
	16.21	2
	16.23	2
	16.29	5
	02.10	3
	02.20	7
	02.40	5
	31.01	3
Total		**43**

Data processing and analysis were performed in Microsoft Excel and *IBM® SPSS® Statistics* (ver. 23), and as statistical methods, descriptive statistics and frequency analysis were used. Descriptive statistics were applied when processing the answers to the questions, which are continuous variables (e.g. number of seasonally employed workers, etc.). Frequency analysis was used to determine the frequency of values of discontinuous variables and their share. Data were processed and analysed for each group of SMEs, depending on the activity of the company: contractors for forestry services, SMEs in primary wood processing and SMEs in final wood processing.

RESULTS

This chapter presents the results, which relate to the characteristics of SMEs in forestry, primary and final wood processing, as well as the organization of their business processes.

CHARACTERISTICS OF SMALL AND MEDIUM ENTERPRISES IN THE JUŽNOKUČAJSKO FOREST REGION

Out of the total number of analysed SME, 44.2% stated that their core business is performing works in forestry. Most of the analysed SMEs in forestry (contractors for forestry services) (98%) were established after 2000 (Table 2).

The cause of this situation, as in other forest areas, is the restructuring of PE “Srbijašume”, which began in 2001. The result of this process was the leasing of the part of the machinery. This enabled private enterprises to start doing certain jobs within the forestry sector. Regarding the number of employees, it is important to note that these are micro-companies that employ less than 10 workers. Of the total number of contractors for forestry services, ¾ employs only one or two workers, while the enterprise with the largest number of employees hires six people. These are mostly family businesses, in which mostly employees are members of the owner's family. Regarding the professional workforce, i.e. forestry engineers and forestry technicians, but also seasonal workers, the research showed that only one enterprise employs a forestry engineer. Based on this, it can be stated that contractors for forestry services lack a professional workforce.

Table 2. Time of SME’s foundation

	Contractors for forestry services	**SMEs in primary wood processing**	**SMEs in final wood processing**
	share (%)		
enterprises founded before 2000	11	20	35,7
enterprises founded after 2000	89	80	64,3
Total	100	100	100

Primary wood processing is the core business for 23.3% of the total number of analysed SMEs. The majority (80%) of **SMEs in primary wood processing** were also established after 2000. This indicates that the process of restructuring PE “Srbijašume” had an impact on the establishment of several enterprises for primary wood processing in this area. Regarding the number of employees, all of them are micro-enterprises, since they do not employ more than 10 workers. In addition, it should be highlighted that 70% have only one employee, while only one company employs 10 workers. As far as the professional workforce is concerned, primary processing enterprises do not employ forestry engineers. This indicates, contractors for forestry services, an obvious lack of skilled labour.

Final wood processing is the core business for 32.6% of the total number of analysed SMEs. The majority (64.3%) of **SMEs in final wood processing** were established after 2000 (Table 2), i.e. after the restructuring of PE “Srbijašume”. In addition, 35.7% of companies were established before 2000, which indicates that SMEs in final wood processing at JKFR have favourable conditions for successful business for a long period. The largest number of enterprises (86%) employ up to 10 workers and belong to the category of micro-enterprises, while the remaining ones, which employ more than 10 workers, can be included in the category of small ones. Related to skilled labour, 21.4% of SMEs in final wood processing employ forestry engineers, while only 7% also employ forestry technicians.

ORGANIZATION OF BUSINESS PROCESSES

The results related to the organization of business processes in SMEs in forestry, primary and final wood processing at JKFR are presented and analysed in this sub-chapter.

Contractors for forestry services at JKFR are performing work in forests both with and without long-term contracts with other enterprises (Figure 1).

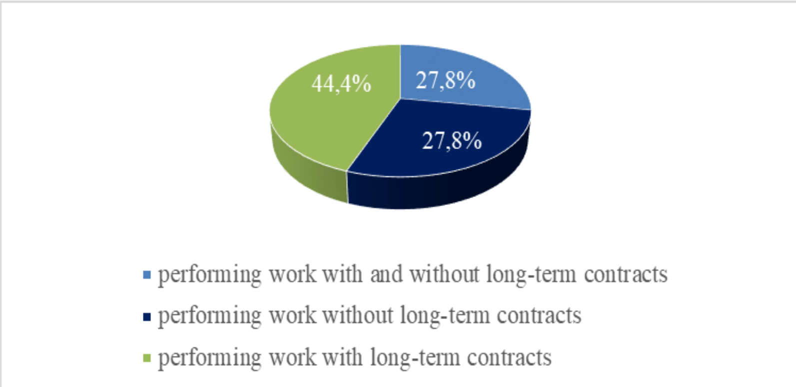


Figure 1. Ways of performing work (SMEs in forestry)

Less than ½ (44.4%) contractors for forestry services perform activities without long-term contracts with other enterprises. In addition, 27.8% of contractors for forestry services perform work based on long-term contracts with other enterprises, which indicates that these SMEs have secured work for a longer period, and thus a stable income. Also, 27.8% of contractors for forestry services perform activities both with and without contracts with other enterprises.

The machinery that contractors for forestry services have, depending on the type of equipment, is shown in Figure 2.

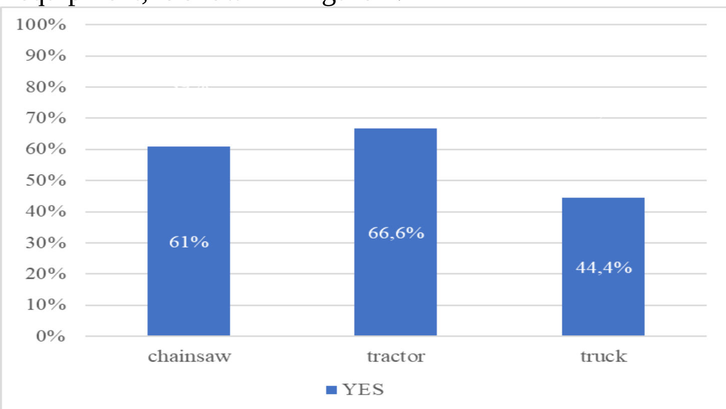


Figure 2. Level of equipment of SMEs with mechanization

More than ½ enterprises own a tractor and a chainsaw, while slightly less than ½ own a forest truck.

The largest number of SMEs (77.8%) perform their activities in state-owned and ⅔ in privately-owned forests. On the other hand, the smallest number of enterprises (Figure 3) performs activities in their own (22.2%), and in church forests (5.6%)[8].

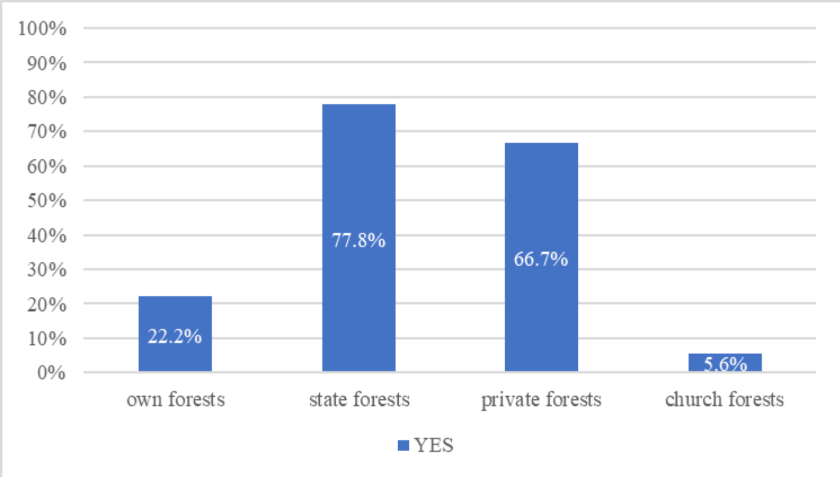


Figure 3. Areas where SMEs operate

Contractors for forestry services perform activities in several previously mentioned categories of ownership. Thus, for example, only five respondents stated that they perform all their activities exclusively in state forests, while only one enterprise performs activities exclusively in privately-owned forests. Based on the previously presented data, it can be said that most companies are directly dependent on jobs in state and private forests.

When it comes to the organization of forest harvesting, 88.9% of respondents said that they perform these works independently (using their own machinery), while only 11.1% of respondents stated that they include other SMEs to help in the realization of work in forest.

The majority (82.4%) of respondents stated that they have no problems at work. Those who face problems during work, point out the obsolescence of mechanization, lack of workers, as well as low labour costs.

Only 11.1% of contractors for forestry services have the facilities for primary wood processing.

The organization of raw material procurement within the **SMEs in primary wood processing** is shown in Figure 4.

[8] Respondents had the opportunity to choose multiple answers, which is why the total is more than 100%.

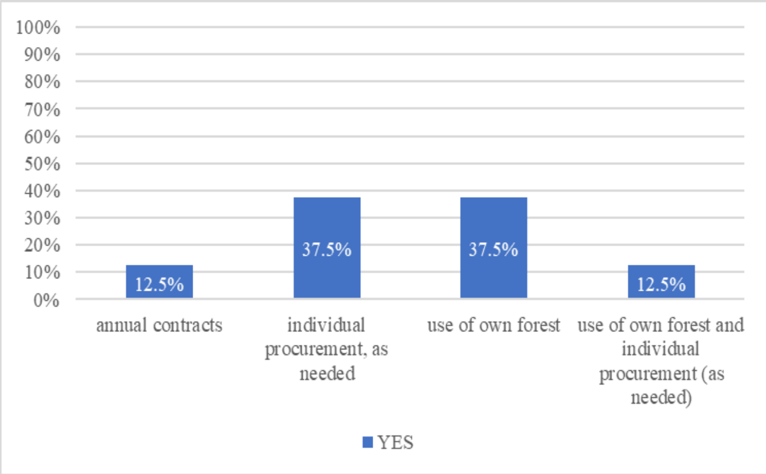


Figure 4. Organization of raw material procurement

Enterprises procure raw materials in several ways:

-based on annual contracts with other enterprises (12.5%);

-through individual procurements, i.e. "as needed" (without long-term contracts with other enterprises) (37.5%);

-using own forest (37.5%).

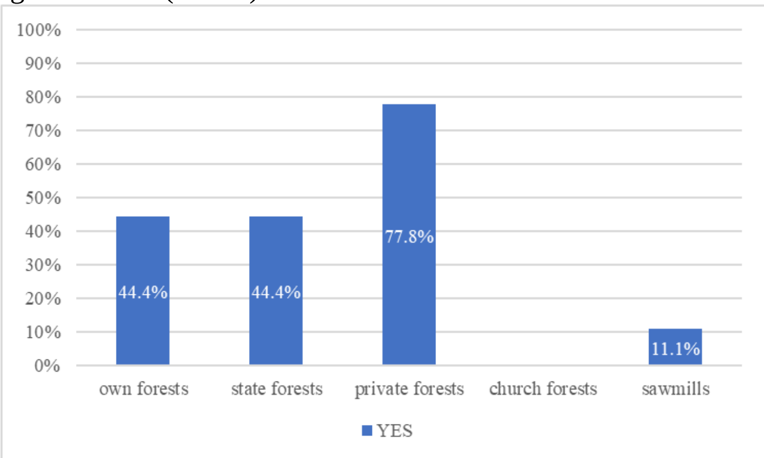


Figure 5. Source of raw materials for SMEs in primary wood processing

In addition, one part of the analysed SMEs (12.5%) procures raw materials in a combination of two ways: by using their own forest, but also, if necessary, from other enterprises.

As problems they encounter when procuring raw materials, the respondents stated the following:

-competition (50%);

-insufficient legal support they need when performing business (50%).

Figure 5 shows the sources of raw materials used by SMEs during the production process.

The largest number of enterprises procure raw materials from private forest owners (77.8%), while the smallest number procure raw materials from sawmills (11.1%). Respondents did not list church forests as one of the sources of raw materials. In addition, it should be emphasized that 33% of respondents procure raw materials exclusively from their own forest property.

The most commonly used tree species is beech (87.5%), followed by other hardwoods (maple, ash, wild cherry), whose share is 55.6%, and oaks (44.4%). The smallest number of enterprises (11.1%) use coniferous tree species as raw material (Figure 6). The large share of enterprises that use beech as a raw material is understandable, considering that this type of tree species is the most widespread at JKFR.

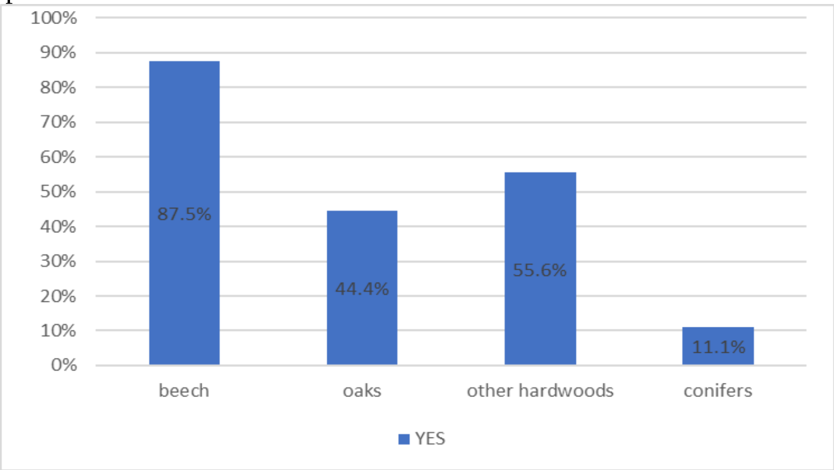


Figure 6. The most commonly used tree species

Most SMEs (77.8%) hire other enterprises to transport raw materials, while the rest use their own means of transport. This indicates that many SMEs are not equipped with appropriate means of transport, which would allow them to deliver raw materials more easily.

Most (88.9%) SMEs have their own capacities when it comes to raw material processing, while others process in cooperation with other enterprises. Only 11.1% of the analysed SMEs in primary wood processing have the facilities for final wood processing.

As the main problems in the processing of raw materials, respondents point out:

-lack of manpower (33.3%);

-lack of mechanization (33.3%);

-inadequate legislative framework, which burdens business (33.3%).

All SMEs in primary wood processing sell their products through retail, after an individual order from customers.

All SMEs in primary wood processing sell their products on the domestic market, while only ⅓ sell them on the foreign market.

The most frequent buyers[9] of products (Figure 7) are individuals, with whom 66.7% of companies cooperate. On the other hand, products are least bought by foreign companies (33.3%). Domestic companies are buyers of products of 44.4% of SMEs for primary wood processing.

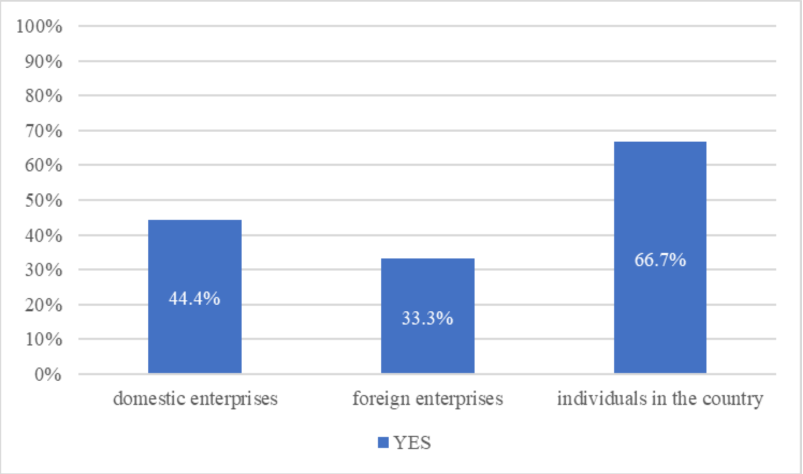


Figure 7. Structure of customers of SME in primary wood processing

The main problems in selling are payment realization (50%) and lack of customers (50%).

SMEs **in final wood processing** procure raw materials in two ways:

1.individual procurement, as needed;

2.annual contracts.

All analysed SMEs in final wood processing procure raw materials through individual procurement. as well, 28.6% have annual contracts with suppliers in addition to individual procurement.

Regarding the sources[10] of raw materials (Figure 8), the majority (78.6%) of SMEs procure raw materials from sawmills, 50% from private forest owners, while the smallest share (21.4%) procure from the state the forest. The large share of raw materials that SMEs in final wood processing procure from sawmills indicates the importance of their connection in the wood supply chain.

These SMEs most often use other hardwoods (78.6%), oak (64.3%) and beech (57.1%) as raw materials[11]. About ⅓ of SMEs in final wood processing (35.7%) use coniferous species as raw material.

[9] Respondents had the opportunity to choose multiple answers, which is why the total is more than 100%.

[10] Respondents had the opportunity to choose multiple answers, which is why the total is more than 100%.

[11] Respondents had the opportunity to choose multiple answers, which is why the total is more than 100%.

Transport of raw materials is organized in two ways: as own transport (42.9%) and through intermediaries (42.9%). One part of the enterprises (14.2%) uses both methods of transporting raw materials to processing facilities.

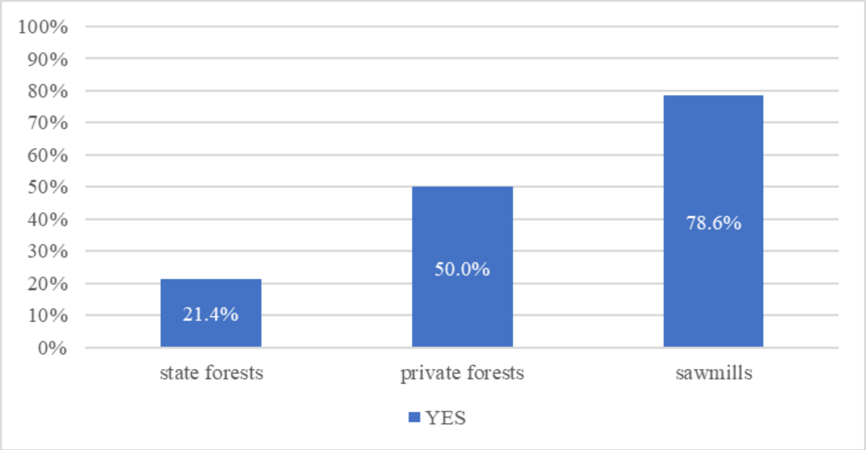


Figure 8. Source of raw materials for SMEs in final wood processing

Most enterprises (78.6%) have their own facilities for processing raw materials, while others process in cooperation with other enterprises. Most enterprises (85.7%) own machines that perform primary wood processing, 64.3% own hand tools, and 92.9% own machines for final wood processing (Figure 9). All respondents stated that lack of manpower is the main problem when processing raw materials.

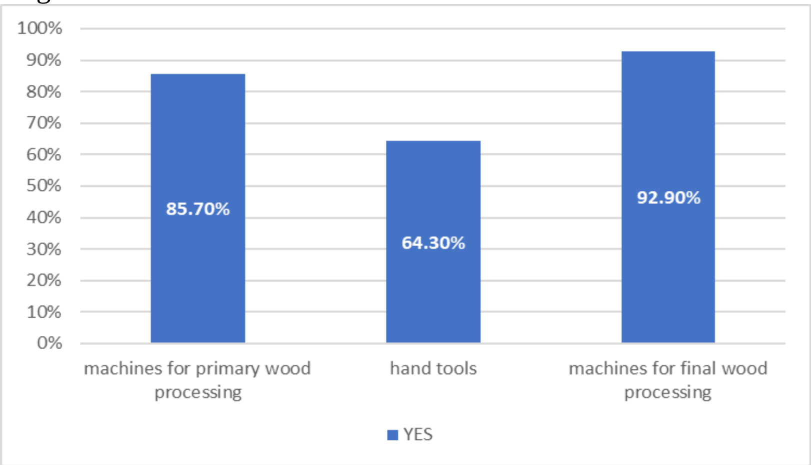


Figure 9. Machines and tools of SMEs in final wood processing

Regarding the way of organizing product selling, the research showed that 78.6% of enterprises sell products through retail, while 21.4% sell products based on annual contracts signed with other companies, in addition to retail.

The majority of respondents (66.7%) believe that the biggest problem with selling is payment realization, while the lack of customers is the problem for 33.3%.

All enterprises place their products on the domestic market, and in addition, 57.1% sell their products abroad.

The structure of customers is shown in Figure 10.

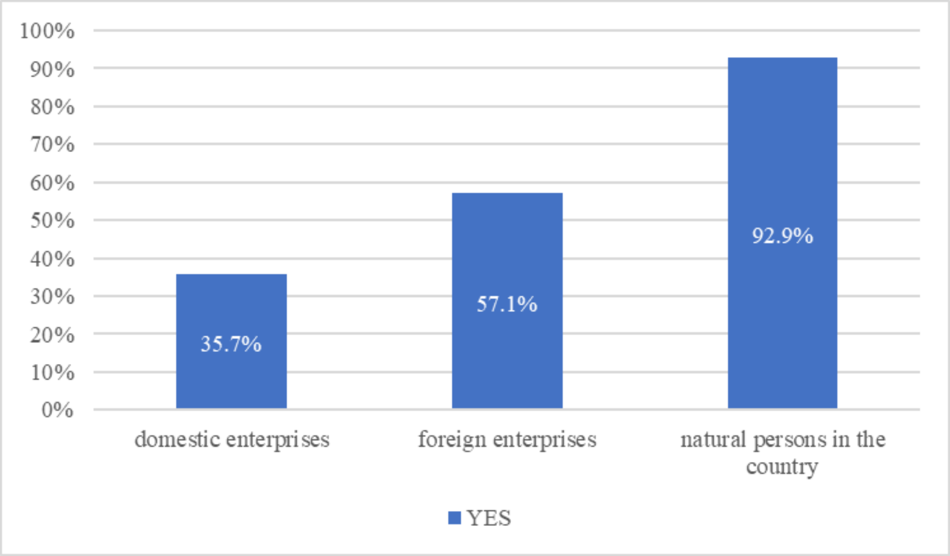


Figure 10. Structure of customers of SME in final wood processing

The majority (92.9%) of enterprises sell products to individuals, while 35.7% sell to domestic enterprises. In addition, 57.1% of SMEs in final wood processing sell to foreign enterprises[12].

DISCUSSION

Regarding the characteristics of the analysed SMEs, the research found that most SMEs in forestry, but also in the wood industry, was established after 2000. These results are in line with previous research in other FRs in Serbia. Namely, a previous survey in the Timočko FR found that 92% of SMEs in the forestry and wood industry were established after 2000 (Ranković et al., 2012). Also, the results of the research conducted in the Južnomoravsko FR show that almost ⅔ SMEs have been established in the last 10 years (Nonić et al., 2018). In the Republic of Srpska, in Banja Luka forestry region, the majority (58%) of enterprises were established after the privatization of part of the state capital in 2001 (Ostić, 2011).

It was found that all enterprises in the forestry sector employ less than 10 workers, which puts them in the category of micro-enterprises, and that most

[12] Respondents had the opportunity to choose multiple answers, which is why the total is more than 100%.

enterprises in the wood industry also fall into this category. The situation is similar in other European countries. For example, ½ out of about 8,000 contractors for forestry service in Poland employ only one worker, about 3,000 employ two to five workers, and only two enterprises have more than 50 employees each. In Germany, it is estimated that there are about 7,300 service enterprises, of which almost ½ have up to five employees including the owner. In Finland, forestry enterprises employ an average of 3.3 workers (Šporčić et al., 2009). In Scotland, it was found that contractors belonging to the category of micro-enterprises in 57% of cases do not have employees, i.e., the only worker is the person who founded the enterprise. When it comes to enterprises from the wood industry, the situation is slightly different, i.e. 35% of enterprises in primary wood processing were without workers (Lawrence, 2018). A survey conducted in Croatia showed that 52.4% of contractors for forestry services do not have permanent employees at the state level. Among enterprises that had permanent employees, only 0.93% employed more than 10 workers (Šporčić & Martinić, 2004). In the Republic of Srpska, in the Vlasenica, Milići, Hanpijesak and Romanija FRs, small enterprises (concerning the number of employed workers) in forestry and wood industry also predominate, with up to 20 employees (Stanišić, 2021). In Serbia, previous research has similar results. Namely, the majority of enterprises (78.6%) in the territory of the Južnomoravsko FR belong to the category of micro and small enterprises (Nonić et al., 2018).

All enterprises, on average, employ less than one forestry engineer and forestry technician. Similar results were found in the research. In Croatia, it has been found that only 2-3% of companies employ forestry engineers (Šporčić & Martinić, 2004). Also, in Južnomoravsko and Timočko FRs, it was found that less than 5% (Nonić et al., 2018) and 9% (Ranković et al., 2012) of the total number of employees have higher education. However, in the Republic of Srpska, in the Vlasenica, Milići, Hanpijesak and Romanija FRs, all contractors for forestry services employ forestry engineers (due to legal regulations, which oblige them to do so), but only ¼ of SMEs in the wood industry have forestry engineers (Stanišić, 2021).

Regarding the organization of business processes, as one of the internal critical success factors of the analysed enterprises, it was noticed that the most common problems encountered by contractors for forestry services at JKFR are: obsolescence of machinery, lack of workers and low labour costs. The situation is similar in Timočko FR, where one of the major problems is the obsolescence of mechanization used when performing various tasks in the forest, regardless of its purpose (Ranković et al., 2012). In the Republic of Srpska, SMEs most often encounter problems related to unused capacity, outdated mechanization and low skill labour force, when performing works on felling, processing, and extraction of wood assortments (Stanišić, 2021).

By transitioning to the form of stable network according to Miles et al (1992), some of these common problems can be diminished. The stable network is designed to serve a mostly predictable market by linking together independently owned specialized assets along with a given product or service value chain. In this network, a large company is managing the assets of its partners, but also taking the responsibility for their output (Miles *et al*., 1992). This "*…stable financial network insulates the firm from market pressures for short-term performance*" (McGuire, Dow, 2009).

When it comes to logging, in Serbia in 2016, about 3.1 million m^3 of wood was felled for commercial purposes, of which the largest share (87.7%) was hardwood, while the rest (12.3%) were conifers (DAS, 2019). Previous research in the Južnomoravsko FR found that 57.1% of enterprises use beech exclusively as raw material, and 21.5%, in addition to beech, most often use oaks, poplars, etc conifers (Nonić et al., 2018). Research presented here shows that 68% of SMEs use beech as raw material, 53.8% oak, 69.2% other hardwoods, such as ash, wild cherry and maple, and 26.9% conifers. It should be taken into account that beech is the most represented tree species in JKFR (present in total volume with 46.5%) (PE "Srbijašume", 2012). That the use of certain types of species for industrial purposes depends on the characteristics of the FR is indicated by the fact that at the level of the Republic of Srpska, 35% of raw materials processed in companies are coniferous trees, most often fir and spruce (Bačić, 2009).

The wood industry enterprises in JKFR most often procure raw materials from private forest owners (65.4%), while in the case of Južnomoravsko FR, about 50% of raw materials come exclusively from the private forests (Nonić et al., 2018). It is important to note that about ⅔ (64%) of the forests on JKFR are privately owned, followed by state-owned forests (32%), while church forests have the smallest share (4%) (PE "Srbijašume", 2012). In addition, it should be added that several enterprises (7.1%) procure raw materials from both private and state forests. It is important to point out that enterprises often do not have only one source of raw materials, but they are procured from a larger number of suppliers.

Only 14.3% of companies in the Južnomoravsko FR have annual contracts with other enterprises for the supply of wood raw materials (Nonić et al., 2018), while at JKFR the number is slightly higher and amounts to 16%.

The largest number of enterprises (92.9%) in the Južnomoravsko FR transport raw materials through intermediaries (Nonić et al., 2018), while in JKFR a much smaller number (50%) use other enterprises for this service. This indicates that the companies at JKFR are better equipped with transport machinery compared to the enterprises from the south of Serbia.

In JKFR, more than ¾ enterprises have facilities for primary wood processing, and more than ½ also have machinery for final wood processing. In the Južnomoravsko FR, 14.3% of enterprises are engaged exclusively in primary processing, while 35.7% perform a lower degree of product finalization (Nonić et al., 2018).

In the period January-June 2019, Serbian companies exported wood, cork, straw and paper worth $728.7 million, which represented 4.4% of total exports at the national level. Products are mostly exported to the EU (51%), followed by countries in the region (26%), but also Russia and Belarus (26%) (DAS, 2019). Data from JKFR partially correspond to the national average, where it was found that about 50% of products are exported exclusively to the EU. None of the respondents stated that they export their products to Russia or countries in the region.

The most common problem faced by enterprises in the Timočko FR when selling is payment realization (72.5%) (Ranković et al., 2012), which is quite similar to the situation with the wood industry in JKFR, where 60% of respondents said that payment realization is the biggest problem. On the other hand, only 28% of contractors for forestry services answered that this is the problem in their business. In the Banja Luka forestry region, 75% of enterprises have a problem with payment realization (Ostić, 2011). In the Vlasenica, Milići, Hanpijesak and Romanija FRs, the payment realization, i.e. illiquidity of customers also stands out as a problem in SME's business (Stanišić, 2021).

To improve the organization of business processes in SMEs in forestry, it is necessary to increase investments in equipment and training of professional staff, perform greater health control and increase health protection of workers, better define legal obligations of entrepreneurs in forestry (Šporčić et al., 2018).

Exernal factors have a significant impact on business success of SMEs (Duarte Alonso & Kok, 2021). For example, JKFR is characterised by bountiful forest resources (JP "Srbijašume", 2012), which makes favourable conditions for entrepreneurship development. According to previous research (Nonić et al., 2012), availability of forest resources, as external factor, is the basic precondition for sustainability of forest-based enterprises. Another important external factor is regulatory framework. This is because SMEs in forestry "…*operate within the context of regulatory frameworks that normalize business and forest management activities*". Very often, these regulations are prohibitive and unstable (Sanchez Badini et al., 2018). However, in previous research in Serbia, which focused on cooperation of forest-based SMEs, it was found that "… *policy framework does not specifically support nor hinder*" it (Nonić et al., 2012). Yet, it must be highlighted that "…*despite the national culture in Serbia does not support entrepreneurship orientation in any single dimension, in SMEs in Serbia entrepreneurial culture is dominant*" (Rodić et al., 2017). However, it was also identified that support measures are an important factor (Dudic et al., 2020), but their implementation in practice is not at high level (Nonić et al., 2012). To accelerate small businesses in forestry, "…*regulatory frameworks should be adequate, simplified, and proportional to the type and size of the enterprises*" (Sanchez Badini et al., 2018). Comparing the European models it was found that the European Union adopts the approach of active participation in the public sector in the development of business activity, particularly the sector of SMEs (Wasilewski, 2015).

CONCLUSIONS

The paper analyses the characteristics of SMEs in forestry and wood industry and the organization of business processes in these enterprises, which have been identified as internal critical success factors.

In relation to the year of establishment, similarities were noticed in all three groups of enterprises, i.e., most SMEs were established after 2000. Based on this, it can be said that the process of reform of PE "Srbijašume", which took place around 2000, significantly influenced the establishment of SMEs in forestry, as well as those dealing with primary and final wood processing.

Based on the number of employees, it can be stated that all enterprises from the forestry and primary wood processing sector belong to the category of micro- enterprises and employ up to 10 workers. The situation with enterprises for final wood processing is a little different, i.e. 14% of them fall into the category of small enterprises, as they employ more than 10 workers.

The analysed SMEs lack a skilled workforce, considering that only one SME from the forestry sector employs an engineer and that primary wood processing enterprises do not employ forestry engineers, while only 20% of final wood processing enterprises in JKFR employed academically educated people.

Almost ½ forestry enterprises work on a contract basis with other enterprises, and this is the case with 20% of final wood processing enterprises, while none of primary wood processing SMEs works on a contract with other enterprises.

In addition, it is important to emphasize that contractors for forestry services perform the largest number of activities in state-owned forests, but also there is a large number of those who directly cooperate with the private forests owners. For example, primary wood processing enterprises usually procure raw materials from private forest owners, while SMEs in final wood processing in most cases procure raw materials from sawmills.

SMEs in primary wood processing most often use beech as a raw material, while in the case of final wood processing enterprises, hardwoods (maple, ash, wild cherry) are most often used. There is a difference between primary and final wood processing enterprises when it comes to selling. Most final processing enterprises (⅔ of them) sell products on foreign markets, while only ⅓ primary processing companies sell their products abroad.

SMEs have problems in their business due to the obsolescence of mechanization, and there is insufficient skilled labour. Also, representatives of SMEs in wood industry see as a problem lack of information regarding export support measures, provided by the public services.

Based on the identified problems, it is **recommended** that, in order to improve internal critical success factors, SMEs should be better informed on the activities and programs of relevant institutions and organizations. According to previous research, such activities and programs belong to the group of external critical success factors. Also, it is necessary to encourage business networking of SMEs in JKFR to jointly sell on foreign markets, which, according to previous research, is also a critical success factor. Providing better information to SMEs and companies networking would have a positive impact on the business of these legal entities.

It is important to point out that the results presented here refer only to the attitudes of SME representatives in the forestry and wood industry in JKFR, because the situation in other regions is not as developed as here and the factors could be different. In that sense, the conclusions and recommendations refer exclusively to contractors for forestry services and SMEs in primary and final wood processing on the territory of JKFR and cannot be directly applied to SMEs in other FRs in Serbia.

For that reason, the recommendations for further research are to conduct such analyses in other FRs, which would determine similarities and differences and give adequate proposals for improving the organization of business processes of SMEs in the forestry and wood industry in Serbia. Also, the recommendation for further research is to define the analytical framework for external critical success factors and their analysis.

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REFERENCES

Aleksić, P. (2021): Javno preduzeće „Srbijašume“ 1991-2021. Beograd: Javno preduzeće za gazdovanje šumama „Srbijašume“ (355)

Alfoqahaa, S. (2018): Critical success factors of small and medium-sized enterprises in Palestine. Journal of Research in Marketing and Entrepreneurship 20(2), 170-188

Banković, S., Medarević, M., Pantić, D., Petrović N. (2009): Nacionalna inventura šuma Republike Srbije-Šumski fond Republike Srbije. Beograd: Ministarstvo poljoprivrede, šumarstva i vodoprivrede-Uprava za šume (244)

Bačić, M. (2009): Entrepreneurship in forestry in the Republic of Srpska: Present Situation, Problems and Development Possibilities, master thesis, Banja Luka: University of Banja Luka (47)

Brumec, J. (2011): Modeliranje poslovnih procesa - Prvi dio: Uvod u modeliranje, Varaždin/Zagreb: KORIS (37)

Chittithaworn, C., Islam, M.A., Keawchana, T., Yusuf, D.H.M. (2011): Factors affecting business success of small & medium enterprises (SMEs) in Thailand, Asian Social Science 7(5), 180-190.

DAS (2019): Forest-based industry in Serbia. Belgrade: Development Agency of Serbia (RAS)

DAS (2021): Forest-based industry in Serbia. Belgrade: Development Agency of Serbia (RAS)

Duarte Alonso, A., Kok, S.K. (2021): Understanding critical success factors and perceived future among micro and small firms through entrepreneurial action theory. European Business Review 33(2), 383-406.

Dudić, B., Dudić, Z., Pejanović, R., Škatarić G., Saxunová D., Peráček T. (2020): The rural policy of Slovakia after joining the EU. Agriculture and Forestry, 66 (3): 33-51.

Eriksson, M., LeBel, L., Lindroos, O. (2015): Management of outsourced forest harvesting operations for better customer-contractor alignment. Forest Policy and Economics 53, 45-55.

Janićijević N., Bogićević Milikić B., Petković M., Aleksić Mirić A. (2019): Organizacija, Beograd: Centar za izdavačku delatnost Ekonomskog fakulteta u Beogradu (674)

Jela (2021): Jela svet- o nama, Retrieved from https://jela.rs/article/jela/o-nama.html

JP "Srbijašume" (2012): Izvod plana razvoja za Južnokučajsko šumsko područje, Beograd: Biro za planiranje i projektovanje u šumarstvu – JP „Srbijašume"

JP „Srbijašume" (2021): Interni izveštaj. Beograd: Javno preduzeće za gazdovanje šumama „Srbijašume"

Kronospan (2021): Organisation, Retrieved from https://www.kronospan-worldwide.com/organisation/

Leidecker, J.K., Bruno, A.V. (1984): Identifying and using critical success factors. Long Range Plan 17(1), 23–32.

Lawrence, A. (2018): Small local forestry businesses in Scotland. Scottish Forestry Journal 72(3), 38-44.

Malhotra, N. (2020): Marketing Research: An Applied Orientation. London: Pearson Education (882)

McGuire, J., Dow, S. (2009): Japanese keiretsu: Past, present, future. Asia Pacific journal of management, 26(2), 333-351.

Miles, R. E., Snow, C. C. (1992): Causes of failure in network organizations. California management review, 34(4), 53-72.

Moore, J. F. (1993): Predators and prey: a new ecology of competition. Harvard business review, 71(3), 75-86.

Moore, J. F. (1998). The rise of a new corporate form. Washington quarterly 21(1), 167-181.

Nedeljković, J. (2021): Organizacija i poslovanje u šumarstvu, praktikum sa radnom sveskom, elektronski izvor, Beograd: Univerzitet u Beogradu-Šumarski fakultet (236)

Nonić, D. (2015): Organizacija i poslovanje u šumarstvu, udzbenik, elektronski izvor, Beograd: Univerzitet u Beogradu-Šumarski fakultet (380)

Nonić, D., Nedeljković, J., Mihajlović, D., Ranković, N., Glavonjić, B. (2018): Organizacija procesa nabavke u lancu snabdevanja drvnom sirovinom: Mogućnosti unapređenja na Južnomoravičkom šumskom podruju. Glasnik Šumarskog Fakulteta 117, 97-118

Nonić, D., Nedeljković, J., Ranković, N., Marinković, M., Glavonjić, P., Weiss G. (2012): Analysis of factors influencing cluster establishment in the Timok forest area in Serbia. Austrian Journal of Forest Science 129(3), 202-227.

Ostić, D. (2011): Organizacija malih i srednjih preduzeća na Banjalučkom šumskoprivrednom području, master rad u rukopisu, Beograd: Univerzitet u Beogradu-Šumarski fakultet (45)

Prasad, D., Pradhan, R., Gaurav, K., Sabat, A (2020): Critical Success Factors of Sustainable Supply Chain Management and Organizational Performance: An Exploratory Study Transportation Research Procedia 48, 327-344.

Ranković, N., Nonić, D., Nedeljković, J., Marinković, M., Glavonjić, P. (2012): Mala i srednja preduzeća u timočkom šumskom području- sistem mera podrške i modela organizovanja, monografija, Beograd: Univerzitet u Beogradu-Šumarski fakultet (265)

Rodić, D., Čudanov, M., Ondrej, J. (2017): Organizational culture of SMEs in Serbia, influence of national culture on entrepreneurial leadership style and philosophy, Journal of entrepreneurship, management and innovation, Proceedings of the 5th international conference: Innovation management, entrepreneurship and sustainability (IMES 2017), 849-859

Sanchez Badini, O., Hajjar, R. Kozak, R. (2018): Critical success factors for small and medium forest enterprises: A review, Forest Policy and Economics 94, 35-45.

Simić, M., Slavković, M., & Stojanović Aleksić, V. (2020). Human Capital and SME Performance: Mediating Effect of Entrepreneurial Leadership. Management:Journal Of Sustainable Business And Management Solutions In Emerging Economies, 25(3), 23-33.

Simpson, M., Padmore, J., Newman, N (2012): Towards a new model of success and performance in SMEs. International Journal of Entrepreneurial Behavior & Research 18(3), 264-285.

Stanišić, M. (2021): Organizacija poslovnih procesa i saradnje malih i srednjih preduzeća na odabranim šumskoprivrednim područjima u Republici Srpskoj, master rad u rukopisu, Beograd: Univerzitet u Beogradu-Šumarski fakultet (102)

Šporčić, M., Bakarić, M., Crnić, I., Landekić, M. (2018): Pregled dobre prakse u šumarskom preduzetništvu. Nova mehanizacija šumarstva 39, 67-82.

Šporčić, M., Martinić I. (2004): Uslužni izvoditelji šumskih radova u Hrvatskoj. Šumarski list broj 11-12, 633-648.

Šporčić, M., Martinić, I., Landekić, M., Lovrić, M., Svakidan, M. (2009): Prikaz stanja preduzetništva u šumarstvu srednje i istočne Evrope. Nova mehanizacija šumarstva 30, 37-46.

Štěrbova, M., Kovalčik, M. (2020): Typology of contractors for forestry services: Insights from Slovakia. Forest Policy and Economics 115, article 102143.

Wasilewski, A. (2015): Efficiency of structural and regional policy measures for development of non-agricultural economic rural activity in selected Polish counties. Agriculture and Forestry, 61(4): 189-196.

Marić,. D. (2022): A preliminary assessment of the ornithological importance of karst poljes at Bosansko Grahovo (Western Bosnia and Herzegovina) - results of a large-scale survey in period 1974 to 2020. Agriculture and Forestry, 68 (1): 95-113. doi:10.17707/AgricultForest.68.1.05

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A PRELIMINARY ASSESSMENT OF THE ORNITHOLOGICAL IMPORTANCE OF KARST POLJES AT BOSANSKO GRAHOVO (WESTERN BOSNIA AND HERZEGOVINA)-RESULTS OF A LARGE-SCALE SURVEY IN PERIOD 1974 TO 2020

SUMMARY

This paper presents the results of long periods of research on the bird fauna of municipality of the Bosansko Grahovo, and thess systematic surveys of the ornithofauna of this area have been running continuously since 1974. This contribution contains a full list of the noted bird species. Birds were recorded at times of nesting, spring and autumn migrations as well as in winter. Twenty years ago, the avifauna of the municipaty of Bosansko Grahovo was poorly known. To date, or to this paper, about 50 bird species have been noted on the municipality of Bosansko Grahovo (Livanjsko polje not included). Birds were recorded at times of nesting, spring and autumn migrations as well as in winter. During large number of fild excursions, which were performed in all seasons, 165 bird species were registered in B. Grahovos area, and today it can safely be said that 105 bird species nest on this area (Livanjsko polje not included). Because of the diversity of its flora and fauna, the area of the municipality of Bosansko Grahovo harbors favorable bird habitats during the whole year.

Key words: Birds, distribution, biodiversity, the municipality of Bosansko Grahovo, karst poljes, types of habitat.

INTRODUCTION

The birds of karst poljes of Bosansko Grahovo (except the part belonging to Livanjsko polje) are poorly researched. Data on the presence of multiple species from Bosansko Grahovo municipality are indicated by Reiser (1939), while data on individual species are available from different authors, such as Fernbach (1963) and Lukač *et al.* (1992). During last few decades, the ornithofauna of karst poljes is intensively researched (Kotrošan *et al.* 2013, 2018; Sackl *et al.* 2014), particularly part of Livanjsko polje (Obratil, 2006; Milanović and Kotrošan, 2012; Stumberger & Sackl 2009), although some poljes are investigated in detail in the past (Puzović *et al.* 2019; Obratil, 1984., 1987.,

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1992). Generally, the area of the municipality of Bosansko Grahovo (=B. Grahovo) is poorly investigated, and in last two decades, smaller and partial researches were conducted on Grahovo's karst poljes (Kotrošan *et al.* 2013 and 2018; Sackl *et al.* 2014) and surrounding mountains (Fernbach, 1963; Hadžiabdić, 2008; Grubešić *et al.* 2011). It is renowned that karst poljes have a great importance for many water-bound bird species, both nesting and migratory (Stumberger and Sackl 2009; Sackl *et al.* 2014; Topić *et al.* 2019). Moreover, it is known that karst poljes represent an important corridor for many European species, particularly during autumn migration (Schneider-Jacoby, 2008; Stumberger & Schneider-Jacoby 2010; Denac *et al.* 2010).

The karst poljes, as well as the complete Dinaric karst are characterized by a diverse, rare and endemic flora and fauna. In particular, the subterranean fauna of the Dinaric Karst is the richest and the most diverse in the world. Grahovo poljes and the whole area of municipality are inhabited by different endemic species (karst endemics), but only the ichthyofauna has been intermittently researched (Marić, 1980, 1983, 1990; Delić *et al.* 2006, etc.).

This paper is aimed to provide a more detailed information on the diversity of birds in this area and its importance for breeding, migratory and wintering birds.

MATERIAL AND METHODS

Study area

Geographical position of Bosansko Grahovo municipality

The study area, the municipality of Bosansko Grahovo, is situated in west part of Bosnia and Herzegovina (B & H) (Fig.1) and it covers 780 km^2. It is situated between 16° 21' 00" and 16° 27' 00" of eastern geographical longitude and 44° 09' 00" and 44° 11' 00" of northern geographical latitude. The municipality of Bosansko Grahovo situated in hilly terrain B&H (Fig. 2) area and surrounded with mountains Uilica (1602 m), Jadovnik (1650 m), Šator (1872 m) and mountain Dinara with its peak Veliki bat (1851 m). There are 4 karst poljes among or between those mountains: Grahovo poljes (3 poljes) are 790-850 m a.s.l., 29 km length and width 2 - 4 km (80 km^2) and Livanjsko polje which is 700-800 m a.s.l. The Livnjsko polje, part of it which belonges to the municipality of Bosansko Grahovo haves surface of around 10.000 ha (average width 8 km, 13 km length). The massif of the Dinara and Uilica Mountain separates the Karst poljes from the Adriatic, which is only 50/60 km away.

In the languages of the Dinaric Karst's countries the term "polje" has different meanings and wide uses. In its broadest way, it "polje" means: flat and open land, often in the sense of living space and the source of goods. However, for the people who live in the Dinaric Karst their most common and peculiar meanings are related to karst poljes (Lučić, 2014).

Different poljes within the area the municipality of Bosansko Grahovo are often inaccurately named in the literature (e.g. Scwarz, 2013; Kotrošan *et al.* 2018; Topić *et al.* 2019; Rubinić *et al.* 2019 etc.).

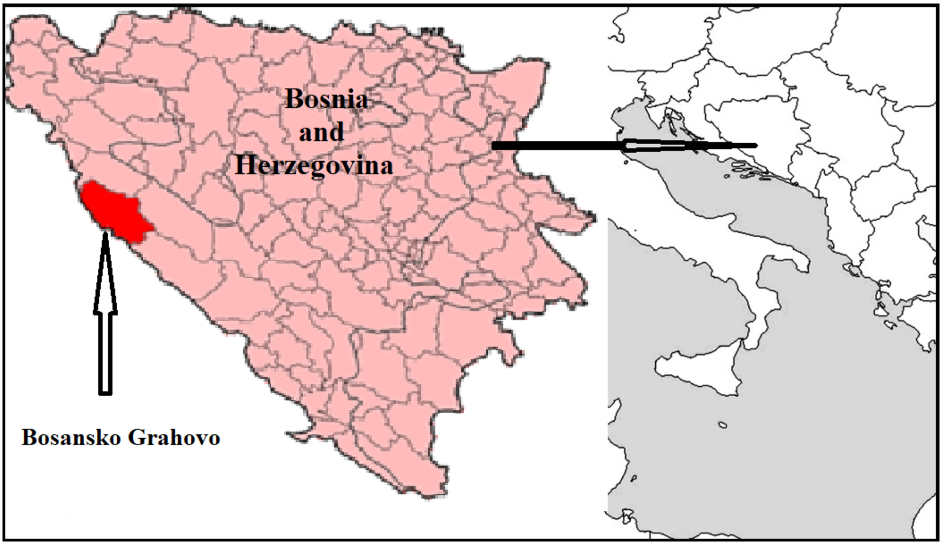

Figure 1. Geographical position the municipality of Bosansko Grahovo

Mostly, all of them are put under name of Grahovo poljes, except the part of Livanjsko polje, as stated in Stumberger et al. (2014). Local people use term “Pašića polje” for flat ground around the town of B. Grahovo and east to Korita village (in length of about 6 - 7 km) as well as the rocky area with many sinkholes on both sides of the road to Livno town. The length of Pašića polje is around 12 km, and its surface area is about 25 km^2. Another polje, situated north-west of the B. Grahovo town towards Drvar is dubbed “Resenovačko polje”, by the similar Resnovci village. Parts of this polje are sometimes named after the nearest village. Besides wrong toponyms, the catchment basins that these poljes belong to are also mis stated (e.g. Stumberger et al. 2014). The waters of Pašića polje, which contain sinking River Korana flow to the Krka River (Adriatic basin), and waters of Resenovačko polje with the sinking River Struga flow to the Unac River, then the Una river, which belongs to the Black Sea basin (Marić, 1980; Bonacci and Ljubenkov 2005; Bonacci *et al.* 2006).

Typical karst poljes shows complex hydrogeological characteristics such as exsurgences, estavelles, swallow holes, and losing rivers. Within the 42 km long valley of B. Grahovo, besides the two mentioned larger poljes (Pašića and Resenovačko polje) there are also smaller polje near villages of Marinkovci and Maleševci at higher altitudes of 790 - 830m. Resenovačko and Pašića poljes are connected through a narrow valley containing numerous karst hollows and depressions.

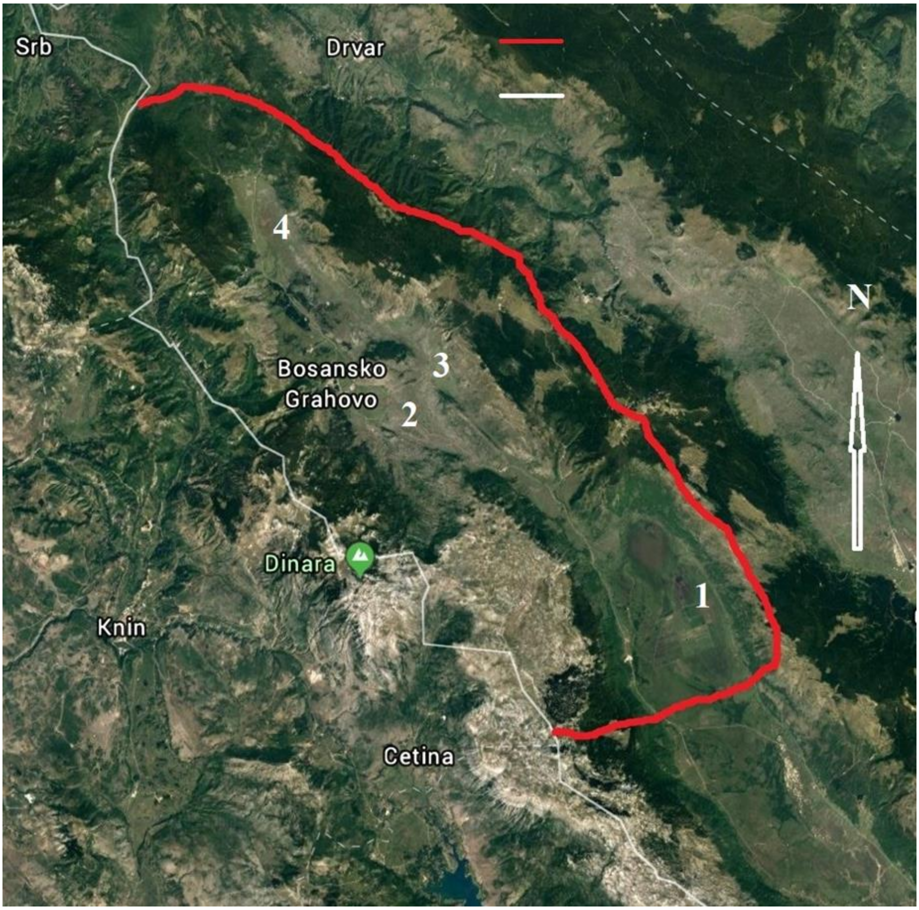

Figure 2. The area of the municipality of Bosansko Grahovo, red line border between the municipalities and white line borders between the states. 1) Livanjsko polje, 2) Pašića polje, 3) Marinkovci polje, 4) Resenovačko polje

Climate at Bosansko Grahovo municipality area

The municipality of Bosansko Grahovo, even though by its position is very close to Adriatic sea, has continental climate with long and sharp winters and short and dry warm summers. Due to its position this area has very big number of sunny days in a year. Mean annual temperature is 5.6 °C and the average annual precipitation is 1,990 millimeters (Federal Hydrome-teorological Institute of Bosnia and Herzegovina).

Demographic Data

At the area of the municipality there are presently 32 settlements (in the past 35) from 550 m a.s.l. up to 1200 m a.s.l. According to the 1991 census, the municipality of Bosansko Grahovo had 35 settlements and 8.311 inhabitants, while today's municipal administration estimates less than 1.000 inhabitants, which shows almost 90% decline in comparison to 1991 census.

Landscapes and biota in the municipality of B. Grahovo

Region of the municipality of B. Grahovo contains all types of karst landforms and features including karren (lapies), dolines, jamas (pits), ponors (swallow holes, sinks), dry and blind valleys, caves and caverns as single forms, and uvalas, poljes and karst plains as larger complex forms.

Characteristic of this area is that poljes are encircled by high hills and mountains (Fig. 2), overgrown with forests on the north side, while southern expositions are barren (pastures) or covered with low forest and bush vegetation. The tallest parts of mountains (above 1500 - 1600 m a.s.l.) are barren pastures, with scarce shrub vegetation (see vegetation types below).

In general, typical karst poljes are elongated and closed depressions with bottoms that have been leveled and covered with arable soils which are surrounded by gentle or, more rarely, by steep mountain slopes. Poljes exhibit complex hydrological and hydrogeological features and characteristics, such as permanent and temporary springs and rivers, losing and sinking rivers (Fig. 3), and swallow holes and estavelles.

Karst poljes are characterized by unique landscape: central parts are flat and usually arable land, and the lower parts, which contain sinking rivers, are swampy and flooded in winter. Situated at the edge of each karst polje there is a small village (Fig. 4), usually by the main roads.

The settlements are surrounded by either small hills or rugged flat land, usually without trees. Those areas are used for grazing (Fig. 5) and they are much larger than arable land.

Figure 3. Sinking River Korana in Pašića polje (photo D. Marić)

Figure 4. Karst polje – the central part of Pašića polje, at the edge of polje there are small villages (photo D. Marić)

Within these pastures, larger dolinas and rock-free patches were also plowed and mowed (Fig. 6). This is the area where flocks of sheep, goats, cows and horses were extensively and seasonally grazed by 60.000 animals in the past. About 50.000 sheep were grazing in the study area year-round, keeping the vegetation here short and succession limited in the past, now about 3.000 (10.000) (personal observation).

The grazing period in the municipality of B. Grahovo typically lasted from mid-March to early July on grasslands and from end-July to early November on hay meadows.

The municipality of Bosansko Grahovo presents a typical livestock region, where arable land (plough-land, gardens, settlements and orchards) covers 4.6%, while forests cover 38.19% of the total area (780 km^2).

Figure 5. Pastures in Pašića polje (photo D. Marić)

Figure 6. A typical *dolina* in arid parts of polje (photo D. Marić)

Permanent pastures and hay meadows cover 57.21% of the total area. Individual ploughs are small, and do not exceed 1 ha. Settlements are either small villages with up to 250 inhabitants or single farms, located between arable land and pastures. Near the settlements, typical land use consists of orchards, a combination of crops and vegetables (autumn and spring-sown cereals, legumes, maize, potatoes, cabbage, beets parsley, carrots, etc.), hay and silage for winter forage, and small grazing pastures for cattle, sheep and horses. Cereals are sown from October to November and harvested in August, but potatoes are sown in April and harvested in September or the first week of October. Potato is currently the most extensively cultivated crop but it covers only about 10% of arable land. After harvesting, the fallow is not ploughed until April. Plough margins are dominated by *Heracleum* spp., *Artemisia* spp., *Achillea* spp., *Cirsium* spp., *Centaurea* spp., and scattered bushes, mostly *Rosa canina* and *Crataegus monogyna*. The meadows are covered by diverse vegetation dominated by *Deschampsia* sp., *Bromus erectus, Brachypodium pinnatum, Calamagrostis villosa*, *Agrostis schraderiana*, *Poa* spp., *Molinia* spp., *Iris* spp., *Ranunculus* spp., *Filipendula* spp., *Hieracium* spp., *Ononis spinosa*, with presence of single of small trees or shrubs (mostly *R. canina, C. monogyna*). Hay is usually mowed before the end of July. Mowing in the B. Grahovo poljes always strongly depended on weather conditions and took place only on days without precipitation. Over the whole observation period, mowing of hay meadows occurred on 30 days.

Natural grazing pastures are an integral part of traditional farmland landscape in karst polje area, where continuous grazing regime sometimes is maintained for centuries. Pasture vegetation is dominated by *B. pinnatum*, *Nardus stricta*, *B. erectus*, *Festuca* spp., *Danthonia* spp., *Stipa* spp. and *Scorzonera villosa*,*Thimus* spp., *Triofolium* spp., *Teucrium* spp., *Eryngium* spp., *Potentilla* spp., *Sanguisorba* spp., *Lotus* spp. The high species diversity of native grasses and herbs is probably a result of specific climatic/weather conditions, geological structures, specific soil nutrient levels etc.

The small height of grasses and herbs was the result from the continuous biomass removal through grazing and no use of artificial fertilizers in the past. Boulders occur frequently within the pastures, which makes the ground unsuitable for plowing and this natural pasture is usually a fine-scaled mosaic of open ground, shrubs, trees, and boulders. The pastures are not privately owned and no boundary hedges exist. According to ecological and vegetation classification of Bosnia and Herzegovina (Stefanović *et al*. 1983), this area belongs to both Mediterranean-Dinaric and Sub-Mediterranean-alpine regions. Folowing sources are used for all descriptions of habitat types: *Interpretation manual of European Union habitats-EUR 28-NATURA 2000*, Brussels: European Commission-DG Environment, http://ec.europa. eu/enviro-nment/nature/legislation/habitats directive/index_en. htm# interpretation. (2013), Fild guide to Natura 2000 habitat types in Bosnia and Herzegovina (Milanović *et al*. 2015).

11 macro habitat types were defined and according to NATURA 2000 twenty-nine habitats types were defined in the municipality of B. Grahovo (not included Livanjsko polje):

1. **ASG** = Alpine and subalpine grasslands or Alpine pastures and stony grasslands (over 1200 meters a.s.l.) and screes (8120, 8140). Four habitat types of pastures were defined (NATURA 2000 code): 6110, 6170, 6230, 62D0.
2. **AB** = Alpine bushes: Bushes with *Pinus mugo* and *Rhododendron hirsutum*., *Pinion mugo* Pawlowski 1928. Three habitat types were defined (NATURA 2000 code): 4060, 4070, 4080.
3. **For** = forest (woods)., deciduous and evergreen, This forest in the municipality of B. Grahovo is represented by several phytocoenoses. Seven habitat types were defined (NATURA 2000 code): 9110, 9130, 9140, 91K0, 91R0, 9410, 9530. There are few relatively preserved forests, more frequent are degraded and sprout-forests in different development stages.
4. **ForW** = Forests along the banks of creeks and rivers, habitat types is probably 91E0 (NATURA 2000 code), these forests (host forests of alder,willows and poplar forests) are along the banks of sinking rivers

(the Struga and the Korana), it is now reduced to fragments and a narrow coastal strip (key species: *Alnus glutinosa, A. incana, Salix alba, S. fragilis, S. purpurea, Populus nigra, P.alba*).

5.HSCH = high shrub deciduous and evergreen bushes (up to 7-10m) or forests with large clearings, degraded forests in different degradation stages (cover 20-40%) (key species: *Fagus silvatica, Acer obtusatum, Sorbus aria, Fraxinus ornus, Carpinus betulus, Betula pendula, Populus tremula, Sesleria autumnalis.*). Two habitat types were defined (NATURA 2000 code): 9140 (*Aceri-Fagetum*-degraded stages*).*, 91K0 (*Ostryo-Fagenion*-degraded stages*).*

6.SCH = deciduous bushes: bushes, shrubs of low trees and shrubs (mostly up to 3m high) with large clearings (20-70%),, Bushes with: *Rossa spp., Crategus spp., Amelanchier ovalis, Corilus avellana, Cornus mas, Cornus sanguinea, Ostrya carpinifolia* (key species) etc, are the common forms of succession, which in the progradation line comes as a developmental stage between pasture and permanent forest stages.

7.DCG = Dry calcareous grasslands, From karst poljes to the montane zone, several types of calcareous grasslands exist. Some of them are on sparse rocky or shingle soils. Three habitat types were defined (NATURA 2000 code): 6110, 6210, 6230.

8.M & Al = Meadow & Agricultural land (arable fields, grasslands and wet meadows of karst poljes). Five habitat types (meadows mowing) were defined (NATURA 2000 code): 62A0, 6410, 6510, 6520, 6540 and arable land. The agricultural soils are mostly sown with wheat, ray, oat, barley, maize, legumes and potatoes.

9.R & C = Rocks and caves, rocky habitats and caves (NATURA 2000 code):8240, 8310.

10.Sett & O = Settlements (villages and town) with Orchards.

11.W (water) **=** Freshwater habitats: springs, brooks, sinking river, short rivers, pools, lakes, wetlands. There is extremely little aquatic vegetation (wetlands) along the water bodies (20-25 ha). mostly by the Lake Pečenci and at the helocrine spring and the Zvijezda stream (both in Pašića polje).

Collection of Field Material

Studies of the ornithofauna of the municipality of Bosansko Grahovo have been running continuously since 1974 to 2020, with the exception of the period 1991 - 2000, when the research was not possible due to civil war and post-war instability. Regular field work was conducted during the whole year, and birds were recorded at times of nesting, spring and autumn migrations as well as in winter. About 2000 days was spent in the field. The used methods were line transect, point counts, "free" method, playback method and capturing and marking method. For *Crex crex* and Strigiformes, night surveys of calling birds were conducted.

The representatives of all species were visually identified using binoculars (8 x 30, 10 x 40 or 9 x 60) and determined using handbooks: Martino and Matvejev (1947) and Peterson *et al,* (1968).

The bird species were classified into three categories based on the species' status as a breeder (nesting N), regular winter visitor (W), or a passage migrant (M) (Table 1.). The order of habitats in the table follows the elevation in the vertical profile.

RESULTS AND DISCUSSION

The results of species' observations in period 1974 - 1991 and 2001 - 2020 from the municipality of Bosansko Grahovo (except Livanjsko polje) are shown in Table 1. During our sampling (all methods), we counted over 100.000 individuals of 163 bird species (Table 1), distributed among the orders of Passeriformes (94), Accipitriformes (9), Piciformes (8), Charadriiformes (7), Galliormes and Pelecaniformes (5), and etc. There are few data about the birds of karst poljes of B. Grahovo municipality, except the belonging part of Livanjsko polje. The best description of the ornithological and natural values of Livanjsko polje is found in the great materials of Ornis Balcanica (Reiser, 1939). Besides data for Livanjsko polje, Reiser (1939) indicates only 34 bird species for the area of the municipality of B. Grahovo, which are not bound to wetlands of Livanjsko polje.

Later, Fernbach (1963), Lukač *et al.* (1992) and few ornithologists in 21 century indicate only a few more bird species for this area. This paper presents the list of 163 species, which is more than 100 species more than mentioned in the available literature (all cited authors). Only two species cited in the literature (Kotrošan *et al.* 2018) were not registered within this research: Spanish Sparrow (*Paser hispaniolensis)* and Glossy Ibis *(Plegadis falcinellus).* Together with 28 species observed by Obratil (2006) at belonging part of Livanjsko polje (Ždralovac), and three more species-Ferruginous Duck (*Aythya nyroca*), Milanović and Kotrošan (2012), Great White Egret (*Ardea alba*), Kotrošan (2018) and Yellow-legged Gull (*Larus michahellis*), Topić (2018), the total number of species recorded in the area of Bosansko Grahovo municipality is 196.

Within the area of B. Grahovo municipality, we registered 105 species that are certain breeders (nests observed) and four species that occur during the breeding season (May or June) but whose nests were not observed. Seven species were occasionally registered during breeding season, and for some, e.g. Collared Flycatcher (*Ficedula albicolis)* or Turtle Dove *(Streptopelia turtur)* there is a suitable breeding habitat. Kotrošan *et al.* (2018) and Rubinić (2019) do not mention European Turtle Dove for Grahovo poljes. Six species were found only on few days in nesting period.

Table 1. Check list of bird species observed in period 1974-1990 and 2001-2020.

Scientific name	Status of species	**Habitats**
Species	N-W-M	
Anas crecca	M sep.	W
Anas platyrhynchos	N	W
Aythiya ferina	M	W
Spatula querquedula	M	W
Alectoris graeca	N	ASG, AB
Coturnix coturnix	N	M & Al
Perdix perdix	N	SCH, DCG, M & Al
Tetrao urogallus	N	For
Tetrastes bonasia	N	For, HSCH,
Columba livia(#)	N	R & C
Columba palumbus (#)	N	For
Streptopelia decaocto	M?	Sett & O
Streptopelia turtur	M?	Sett & O
Cuculus canorus	N	AB, For, HSCH, SCH, Sett & O, ForW
Apus apus(#)	N	R & C
Grus grus	M	M & AL
Podiceps cristatus	M	W
Podiceps nigricollis	N?	W
Tachybaptus ruficollis	N	W
Crex crex	N	M & AL
Gallinula chloropus	N	W
Fulica atra	N	W
Porzana porzana	In May	W
Rallus aquaticus	In May	W
Ciconia ciconia	M	M & AL
Ardea cinerea	?	W
Ardea purpurea	M	W
Ardeola ralloides	M	W
Botaurus stellaris	N?	W
Nycticorax nycticorax	In May	W
Vanellus vanellus	?	W
Scolopax rusticola	N	HSCH, SCH, ForW
Actitis hypoleucos	N	W
Gallinago gallinago	?	W
Tringa glareola	?	W
Tringa nebularia	M sep	W
Tringa ochropus	N?	W
Gyps fulvus #	EX	/
Accipiter gentilis(#)	N	For
Accipiter nisus(#)	N	For
Aquila chrysaetos (#)	N	R & C, For
Clanga pomarina	M (1)	N.N.
Buteo buteo (#)	N	For
Buteo lagopus	Octob.	DCG
Circus aeruginosus(#)	?	M & AL
Circus pygargus	N?	DCG, M & AL
Circaetus gallicus(#)	N	N.N.
Athene noctua (#)	N	R & C
Asio otus(#)	N	For
Bubo bubo	N	For
Otus scops(#)	N	Sett & O, ForW
Strix aluco	N	For
Upupa epops	N	R & C, DCG, Sett & O, ForW

Merops apiaster	In June	DCG
Alcedo atthis	N	ForW, W
Jynx torquilla	N	For, HSCH, Sett & O, ForW
Dendrocopos leucotos	N	For, HSCH, Sett & O
Dendrocopos major	N	For, Sett & O, ForW
Dryobates minor	N	For, HSCH,
Dryocopus martius	N	For
Dendrocoptes medius	N	For, HSCH,
Picus canus	N	For
Picus viridis	N	For, Sett & O
Falco biarmicus	M/May	M & AL
Falco naumanni	M/Sep	DCG, M & AL
Falco subbuteo(#)	N	For
Falco tinnunculus(#)	N	R & C, For, Sett & O
Falco vespertinus	M/Sep-Okt	M & AL
Oriolus oriolus	N	For, HSCH, Sett & O, ForW
Corvus corax(#)	N	R & C, For
Corvus cornix(#)	N	For, Sett & O, ForW
Corvus frugilegus	M	M & AL
Coloeus monedula	W	M & AL
Garrulus glandarius	N	For, HSCH, Sett & O
Nucifraga caryocatactes	N	For, Sett & O
Pica pica (#)	N	Sett & O, ForW
Pyrrhocorax graculus	N	ASG, R & C
Lanius collurio	N	HSCH, SCH, DCG, M & Al, Sett & O, ForW
Lanius excubitor	W	Sett & O, M & Al, ForW
Lanius minor	N	Sett & O, M & Al, ForW
Lanius senator	M/May	SCH
Parus major	N	For, HSCH, Sett & O, ForW
Poecile montanus	N	For
Poecile palustris	N	For, HSCH
Periparus ater	N	For
Cyanistes caeruleus	N	For, HSCH,
Alauda arvensis	N	ASG, DCG, M & Al
Galerida cristata	W	DCG, M & Al
Eremophila alpestris	N	ASG
Lullula arborea	N	SCH, DCG, Sett & O
Aegithalos caudatus	N	For, HSCH, ForW
Delichon urbicum(#)	N	Sett & O
Hirundo rustica(#)	N	Sett & O
Acrocephalus arundinaceus	N	W
Acrocephalus paludicola	M	W
Acrocephalus scirpaceus	N	For, W
Hippolais icterina	M	Sett & O
Hippolais olivetorum	M	Sett & O
Phylloscopus collybita	N	AB, For, HSCH
Phylloscopus sibilatrix	N	For, HSCH
Phylloscopus trochilus	M	For, HSCH, Sett & O, ForW
Sylvia atricapilla	N	For, HSCH, SCH, Sett & O, ForW
Sylvia borin	M	Sett & O
Curruca communis	N	HSCH, SCH, Sett & O
Curruca curruca	M	HSCH, SCH, Sett & O
Curruca hortensis	M	HSCH, SCH, Sett & O
Curruca nisoria	N	HSCH, SCH, Sett & O
Regulus ignicapillus	M	For, HSCH, Sett & O
Regulus regulus	N	AB,For
Bombycilla garrulus	W	SCH, Sett & O, M & Al, ForW,

Sitta europaea	N	For, HSCH, Sett & O,
Sitta neumayer	N	ASG, R & C
Certhia brachydactyla	?	Sett & O
Certhia familiaris	N	For
Troglodytes troglodytes	N	For, HSCH, Sett & O
Sturnus vulgaris(#)	N	Sett & O, ForW
Cinclus cinclus	N	W
Turdus iliacus	M (Oct.)	Sett & O
Turdus merula	N	For, HSCH, SCH, Sett & O, ForW
Turdus philomelos	N	For
Turdus pilaris	W	SCH, M & Al, Sett & O, ForW
Turdus viscivorus	N	For, HSCH, SCH, Sett & O, ForW
Muscicapa striata	N	HSCH, SCH
Ficedula albicolis	M?	HSCH, SCH, Sett & O
Ficedula hypoleuca	M	Sett & O, ForW
Erithacus rubecula	N	For, HSCH, SCH, Sett & O
Luscinia luscinia	M	Sett & O
Luscinia megarhynchos	N	Sett & O, ForW
Monticola saxatilis	N	ASG, R & C, DCG
Oenanthe hispanica	M	DCG
Oenanthe oenanthe	N	ASG, DCG
Saxicola rubetra	N	DCG, M & Al
Saxicola rubicola	M	M & AL
Phoenicurus phoenicurus	In June	HSCH, SCH, Sett & O
Phoenicurus ochruros	N	ASG, R & C, Sett & O
Petronia petronia	M Sep.	SCH, DCG
Prunella collaris	N	ASG, AB
Prunella modularis	N	ASG, For
Passer domesticus(#)	N	Sett & O
Passer montanus	W	Sett & O, ForW
Anthus campestris	N	DCG
Anthus pratensis	M/Okt.	M & AL
Anthus spinoletta	N	ASG
Anthus trivialis	N	HSCH
Motacilla alba(#)	N	R & C, Sett & O
Motacilla cinerea	N	W or ASG
Motacilla flava	N	M & AL
Linaria cannabina(#)	N	AB, SCH, DCG
Carduelis carduelis(#)	N	For, HSCH, Sett & O, ForW
Spinus spinus	N	For
Chloris chloris	N	For, HSCH, Sett & O, ForW
Coccothraustes coccothraustes	N	For, HSCH, Sett & O, ForW
Fringilla coelebs	N	For, HSCH, Sett & O, ForW
Fringilla montifringilla	W	Sett & O, M & Al
Loxia curvirostra(#)	N	For
Pyrrhula pyrrhula (#)	N	For
Serinus serinus	N	For, Sett & O
Emberiza cia	N	HSCH, SCH
Emberiza citrinella	N	For, HSCH, SCH, Sett & O, ForW
Emberiza hortulana	N	SCH
Emberiza melanocephala	N	SCH, Sett & O
Emberiza schoeniclus	M	ForW
Miliaria calandra	N	DCG, Sett & O, M & Al, ForW

(period/season: W=wintering, M=migration, N=nesting-breeding., habitats: ASG, AB, For, ForW, SCH, DCG, M & Al, R & C, Sett & O, W, see above in methods., (1) = species observed at least once).

(#)Species detected during the breeding period outside of breeding habitat (mostly while feeding).

On the Dinara Mountian, Grifon Vulture *(Gyps fulvus*) used to breed until 1968. One poisoned individual was brought by hunters to Luka village in May 1969 (personally witnessed). It is generally known that this species has disappeared from the municipality of B. Grahovo 50 years ago. Single individuals of *A. chrysaetos* and *A. gentilis* can be regularly observed during the all season and one individual of *C. pomarina* was observed once near the Korana Rivr (May 20, 1982). Single individuals or pairs of *C. aeruginosus* and *C. pygargus* can be seen in the nesting season (April, May, July and August) but these individuals do not nest here, they are vagrants. Acording to Rubinić *et al.* (2019) *C. pygargus* breeds in Grahovo poljes. Also, on the Dinara Mountains, in the municipality of Bosansko Grahovo, the threatened shore lark (*E. alpestris*) was found in only 3 breeding pairs. This threatened species is also rare in the neighboring mountains (Lukač *et al.* 2017).

Due to limited wetland area, breeding water birds are rare, e.g. Moorhen *(G. chloropus)* and Coot *(F. atra)*, with 1 or 2 pairs in Pečenačko Lake. Common Sandpiper *(A. hypoleucos)* breeds irregularly in this area.

Dendrogram shows that diversity of breeding birds in the habitat Set & O is similar to forest habitats including shrub lands, and the similarity is the greatest with habitat ForW (see Fig. 7). The most similar habitats by breeding birds' diversity are pastures and arable fields (M & Al).

During migration period, 33 species of non-breeding birds were registered, mostly at autumn migration (September). Multiple species, like warblers *Hippolais* spp., *S. borin* or *C. curruca*, were registered over a longer period, especially species that feed on fruits and berries and some species were registered over only few days in both periods, e.g. Common Crane *(G. grus)*, Night Heron *(N. nycticorax)*, White Stork *(C. ciconia)*, *etc*. Rubinić (2019) and Rubinić *et al*. (2019) do not indicate Common Crane in this area during migration, although this species is sometimes present in larger numbers (about 50 individuals). Topić and Topić (2017) mention only one individual at Pašića polje. The falcons, *F. naumanni* and *F. vespertinus*, were sporadic autumn migrant (very rare) while *F. biarmicus* was sporadic spring migrant (in May). Rubinić (2019) and Rubinić *et al*. (2019) indicate the presence of *F. naumanni* in larger numbers, and *F. vespertinus* as scarce. The Rough-legged Buzzard (*B. lagopus)* was registered only in two years: three specimen in 2011 and four specimen in 2020 on Pašića polje. Water birds (W) are generally scarce during migration. During autumn migration, mass flocks are formed by Starlings, and Meadow Pipits occur with few hundred individuals in October. In the past, large flocks were formed by seed-eating species, including *F. montifringila* which is present also during the winter in this area. Regular and numerous wintering species (WV – winter visitor) are Brambling *(F. montifringilla)* and Fildfare *(T. pilaris)*, and in much smaller numbers also Great Grey Shrike (*L. excubitor)* and Crested Lark *(G. cristata)*. Ocassionally, during strong winters, this area is attended by Waxwing *(Bombycilla garrulus)* where it feeds on *Crataegus* spp. and *Rosa* spp.

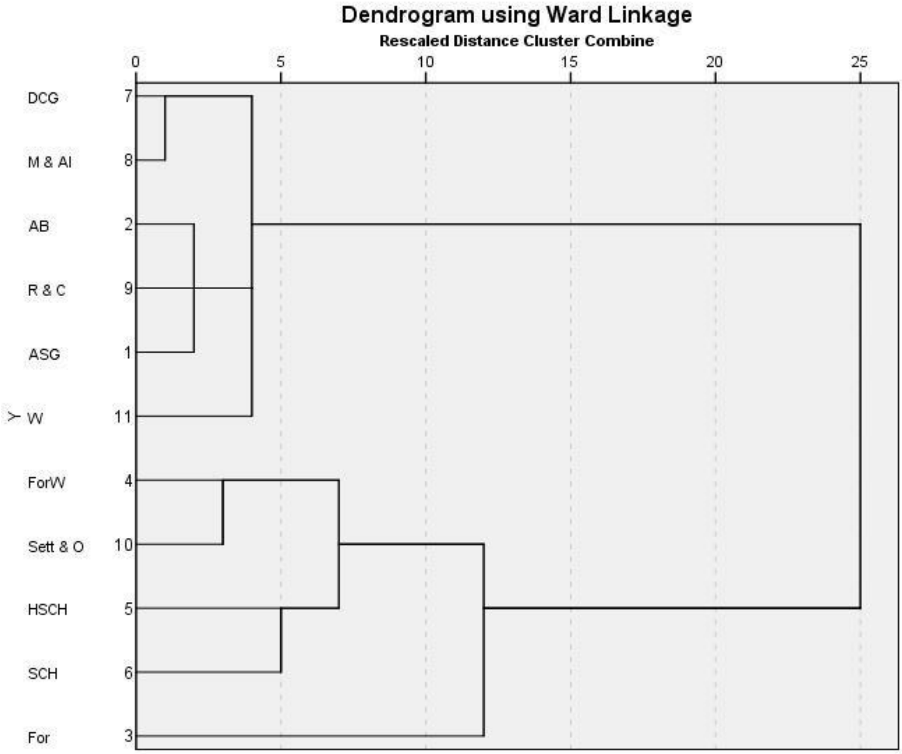

Figure **7**. Dendrogram of breeding birds' diversity in 11 habitat types

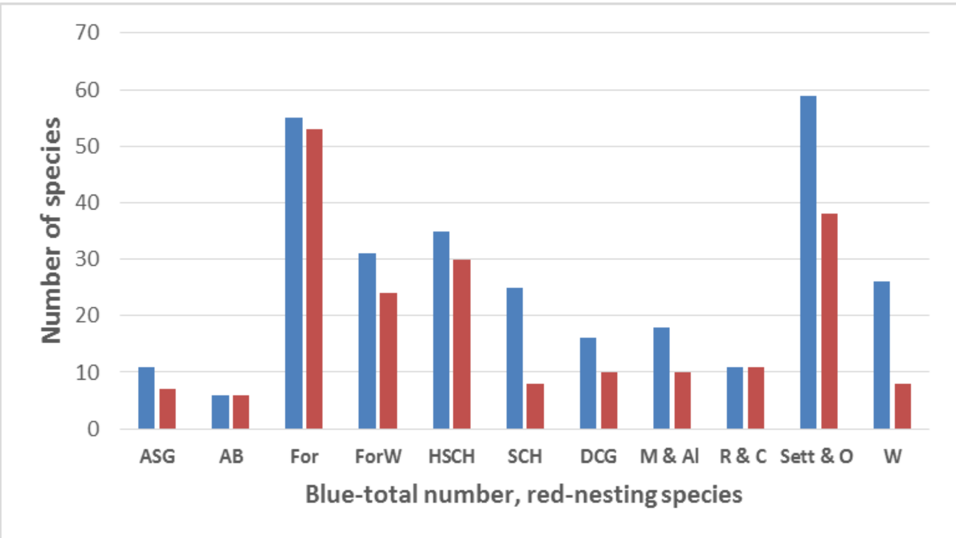

Figure 8. Number of species in 11 types of habitats in the area of Bosansko Grahovo municipality

Of all wintering visitors, the earliest one is *L. excubitor*, which arrives in the first half of October, after the first substantial cold (in Europe), and other species mostly arrive much later.

The majority of breeding species was registered in forest habitats (For), although the greatest number of species is found in settlments and orchars (Sett & O). Total number of species and number of breeders by habitat type is presented on Figure 8.

Alpine and subalpine ecosystems had the fewest bird species. The graph 8 shows that wetlands, settlements and orchards, and then progradation stadium of shrub lands (SCH) have the greatest importance for migratory species. Since the wetland habitats (W) are small, they provide suitable resources only for smaller flocks or just few individuals. The orchards, besides being important for many migratory species, attract many species during the breeding season, especially during cherry ripening, even if they are not characteristic for such habitat. Similar observations are brought by Holmes and Robinson (1981). In general, a small number of bird species e.g. Rock partridge *(A. graeca),* Capercailie *(T. urogallus),* Hazel Grouse *(T. bonasia)* and some others, do not depend on karst poljes habitats (ForW, DCG, M & Al, Sett & O, W), including settlements with orchards.

Karst poljes in the municipality of Bosansko Grahovo are important centers of biodiversity in Bosnia and Herzegovina and they are of great importance for bird fauna. For certain species, such as Corncrake (*Crex crex*), Lesser Grey Shrike (*L. minor*), and others, karst poljes are important nesting sites. On the other hand, for a big number of species Grahovo poljes are of great importance during migration periods. More than 100 species migrate through this area.

In the spring, particularly numerous are flycatchers (Muscicapidae). The last species that leave this area in larger flocks (over 50 individuals) in late October are *P. ochruros*, *S. torquata* and *A. pratensis* usually with more than 100 individuals. This conclusion is supported by the recently identified bird migration path called the “Adriatic Flyway”.

CONCLUSIONS

The paper corrects incorrect names for the studied karst poljes (fields). This paper to provide a more detailed information on the diversity of birds in this area and its importance for breeding, migratory and wintering birds. 165 bird species were registered in B. Grahovos area, and today it can safely be said that 105 bird species nest on this area (Livanjsko polje included). We have registered several endangered bird species that nest or migrate in this area. Because of the diversity of its flora and fauna, the area of the municipality Bosansko Grahovo harbors favorable bird habitats during the whole year.

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REFERENCES

Bonacci, O. & Ljubenkov, I. (2005): New insight into the Krka River Hydrology. *Croatian Waters* 52: 265–281 (in Croatian).

Bonacci, O., Jukić, D. & Ljubenkov, I. (2006): Definition of catchment area in karst: case of the rivers Krčić and Krka, Croatia. *Hydrological Sciences–Journal–des Sciences Hydrologiques*, 51(4): 682-699.

Delić, A., Kučinić, M, Marić, D. & Bučar, M. (2005): New data about the distribution of *Phoxinellus alepidotus* (Heckel, 1843) and *Aulopyge huegelii* (Heckel, 1841). *Natura Croatica,* 114(4): 351-355.

Denac, D., Schneider-Jacoby, M. & Stumberger, B. (eds.) (2010): Adriatic flyway – closing the gap in bird conservation. Euronatur, Radolfzell- http://www.euronatur.org/Facts-and-Figures.936.0.html.

Fernbach, J. (1963): Ornitološke bilješke s Dinare i Šator planine. *Larus,* 15: 210-211.

Grubešić, M., Tomljanović, K., Kunovac, S. (2011): Rasprostranjenost i brojnost jarebice kamenjarke grivne (*Alectoris graeca* Meisner) u Hrvatskoj i Bosni i Hercegovini. *Šumarski list,* 11-12: 567-574.

Hadžiabdić, S. (2008): Stanje populacija velikog tetrijeba (*Tetrao urogallus* L.) na području Federacije Bosne i Hercegovine. *Ornitološko društvo "Naše ptice" Sarajevo,*: 68-71.

Holmes, R.T. & Robinson, S.K. (1981): Tree species preferences of foraging insectivorous birds in a northern hardwoods forest. *Oecologia*, 48: 31–35.

Interpretation manual of European Union habitats-EUR 28-NATURA 2000, European Commission DG Environment, Nature ENV B.3., 2013 Brussels: http://ec.europa. eu/environment/nature/legislation/habitats directive/index_en. htm# interpretation

Kotrošan, D., Dervović, I., Topić, G., Drocić, S., Gotovac, M., Šarac, M. & Šarčević, I. (2013): Rezultati prvog popisa sivog svračka (Lanius minor) na kraškim poljima Bosne i Hercegovine. *Bilten Mreže posmatrača ptica u Bosni i Hercegovini*, 9(9): 40-46.

Kotrošan, D., Sarajlić, N. Topić, G., Radoš, D. Topić. B. & Šarac. M. (2018): *Priručnik za edukaciju turističkih vodiča za promatranje ptica na krškim poljima Bosne i Hercegovine*. Sarajevo-Tomislavgrad, 168 pp.

Lučić, I. (2014): General aspects of the Karst Poljes of the Dinaric Karst. In: Sackl P., Durst R., Kotrošan D. & Stumberger B. (eds.), Dinaric Karst Poljes-Floods for Life. *EuroNatur, Radolfzell*, pp 17-24.

Lukač, G., Milovac, M., Bušljeta, N., Adžić, I., Andačić, N., Vujčić-Karlo, S., Stani, W. (2017): Velebitske ptice. Senj. zb. 44: 47-72.

Lukač, G., Stipčević, M., Crnković, R. & Bem, D. (1992): Characteristics of habitat and distribution of *Sitta neumayer* Mich. (Aves) in Croatia and neighbouring areas. *Natura Croatica*, 1: 81-91.

Marić, D. (1980): Prilog poznavanju rasprostranjenja *Paraphoxinus alepidotus* (Heckel, 1843) u vodama Jugoslavije. *Glasnik Republičkog Zavoda za zaštitu prirode-Prirodnjačkog muzeja,* 13: 101-105.

Marić, D. (1983): Morfološke karakteristike *Paraphoxinus alepidotus* (Heckel, 1843) (Cyprinidae) u ponornici Korani kod Bosanskog Grahova. *Glasnik Republičkog Zavoda za zaštitu prirode-Prirodnjačkog muzeja*, 16: 67-73.

Marić, D. (1990): Razmnožavanje *Phoxinellus alepidotus* Heckel, 1843 iz ponornice Korane kod Bosanskog Grahova. *Glasnik Republičkog Zavoda za zaštitu prirode-Prirodnjačkog muzeja*, 23: 75-84.

Martino, B.E. & Matvejev, S.D. (1947): *Ptice Jugoslavije (skupljanje, prepariranje i određivanje).* Prosveta, Beograd, 222 pp.

Milanović, Đ. & Kotrošan, D. (2012): *Ptice i šaševi Livanjskog polja – priručnik za praćenje stanja šaševa (Carex sp.) i indikatorskih vrsta ptica u širem području Ždralovca.* Centar mladih Livno i Ornitološko društvo „Naše ptice" Sarajevo, 124 pp.

Milanović, Đ., Brujić, J., Đug, S., Muratović, E. & Lukić Bilela, L. (2015): *Fild guide to Natura 2000 habitat types in Bosnia and Herzegovina*. Prospect C&S s.a. Rue du Prince Royal 83, 1050 Bussels, Belgium,189 pp.

Obratil, S. (1984): Naselja ptica (Aves) u kopnenim biocenozama kraških polja Hercegovine. *Glasnik zemaljskog muzeja Bosne i Hercegovine* (PN) NS, 23: 147-184.

Obratil, S. (1987): Naselje ptica u ekosistemima Gatačkog polja i okoline prije izgradnje TE Gacko. *Glasnik zemaljskog muzeja Bosne i Hercegovine* (PN) NS, 25-26: 211-237.

Obratil, S. (1992): Prilog poznavnja ornitofaune buškog jezera (Bosna) i užeg priobalnog područja. *Glasnik zemaljskog muzeja Bosne i Hercegovine* (PN) NS, 30: 155-168.

Obratil, S. (2006): Ekološka diferencijacija faune ptica Livanjskog polja (Bosna). *Glasnik zemaljskog muzeja Bosne i Hercegovine* (PN) NS, 32: 45-81.

Peterson, R., Mountfort, G. & Hollom, P.A.D. (1968): *Die Vogel Europas (achte auflage*. Verlag Paul Parey, Hamburg und Berlin, 417 pp.

Puzović, S., Vučković, V., Stojnić N., Sekulić, G., Radaković, M., Dučić, N., Rudić, B., Ružić M., Radišić D., Grubač, B., Šćiban, M., & Raković, M. (2019): Birds of Pešter karst polje. In: Sackl, P., Ferger, S., Sarajlić, N., Kotrošan D. & Topić, G. (eds).Dinaric Karst Poljes-Nature Conservation and Rural Development. *Ornitološko društvo "Naše ptice" Sarajevo*, 51-85

Reiser, O. (1939): *Materialien zu einer Ornis balcanica. Band I, Bosnien und Herzegowina*. Annalen des naturhistorischen Museums, Wien.

Rubinić, B. (2019): Karst poljes in Bosnia and Herzegovina – newly identified Important Bird Areas In Sackl,P., Ferger, S. W., Sarajlić, N. Kotrošan, D., Topić, G. (eds): Dinaric Karst Poljes –Nature Conservation and Rural Development. *Ornitološko društvo „Naše ptice"* Sarajevo, pp 123-139.

Rubinić, B., Sjeničić, J., Sackl, P., Kotrošan, D. & Sarajlić, N. (2019): List of karst poljes in Bosnia and Herzegovina proposed as new Important Bird Areas. In Sackl, P., Ferger, S. W., Sarajlić, N. Kotrošan, D., Topić, G. (eds): Dinaric Karst Poljes –Nature Conservation and Rural Development. *Ornitološko društvo „Naše ptice"* Sarajevo pp 143-192.

Sackl, P., Dervović, I., Kotrošan, D., Topić, G., Drocić, S., Šarac, M., Sarajlić, N., Durst, R., & Stumberger, B. (2014): The distribution and population numbers of Corncrakes *Crex crex* in the karst poljes of Bosnia and Herzegovina-results of a large-scale survey in 2012 and 2013. In: Sackl, P., Durst, R., Kotrošan, D. & Stumberger, B. (eds.), Dinaric Karst Poljes-Floods for Life. EuroNatur, Radolfzell, pp 91-103.

Schneider-Jacoby, M. (2008): How many birds migrate over the Adriatic Sea? – *Acrocephalus*, 29: 1−3.

Scwarz, U. (2013): »Flooding analysis of Karst Poljas in Bosnia & Herzegovina«. Dunaj: FLUVIUS. Http://www.euronatur.org/fileadmin/docs/docs_english/ *Karst-workshop October 2013 /Flooding_analysis_of_Karst_Poljes_in_Bosnia_and_Herzegovina_3152013.pdf.*

Stefanović, V., Beus, V., Burlica, Č., Dizdarević, H., & Vukorep, I. (1983): *Ekološko-vegetacijska rejonizacija Bosne i Hercegovine*. Posebna izdanja 17, Šumarski fakultet u Sarajevu. 62 pp, Sarajevo.

Stumberger, B. & Schneider-Jacoby, M. (2010): International importance of three Adriatic Flyway priority sites: Livanjsko Polje, the Neretva Delta and Lake Skadar-Shkoder with the Bojana-Buna Delta. In: Denac, D., Schneider-Jacoby, M. & Stumberger, B. (eds.) (2010): Adriatic flyway – closing the gap in bird conservation. EuroNatur, Radolfzell, pp 53-58.

Stumberger, B. & Sackl, P. (2009): Results of the waterbird counts and the breeding status of waterbirds in Livanjsko polje, 2007 – 2009. *Bilten mreže posmatrača ptica u Bosni i Hercegovini*, 4-5(4-5): 38–54.

Stumberger, B., Durst, R., Kotrošan, D. & Mulaomerović, J. (2014): List of karst poljes in Bosnia and Herzegovina Ed: Sackl, P., Durst, R., Kotrošan, D. and Stumberger, B. Dinaric Karst Poljes – Floods for Life, pp 129-196.

Topić, B. & Topić, G. (2017): Glossy Ibis (*Plegadis falcinellus* Linnaeus, 1766) in Pašića polje. *Bilten mreže posmatrača ptica u Bosni i Hercegovini*, br. 13:71.

Topić, G., (2018): Results of the International Waterbird Census in Bosnia and Herzegovina in 2018. *Bilten mreže posmatrača ptica u Bosni i Hercegovini*, 14: 10-39.

Topić, G., Topić, B., Kotrošan, D., Šarac, M. & Vekić, J. (2019): Analysis of the occurrence of Lesser Kestrel (*Falco naumanni*) and Red-footed Falcon (*Falco vespertinus*) in the karst poljes of Bosnia and Herzegovina in the 2012-2017 period. In Sackl, P., Ferger, S.W., Sarajlić, N. Kotrošan, D., Topić, G. (eds): Dinaric Karst Poljes –Nature Conservation and Rural Development. *Ornitološko društvo „Naše ptice“* Sarajevo, pp 93 – 112.

Ganić, A., Begić, M., Forto, A., Krvavica, M. (2022): Determination of quality parameters of Herzegovinian dry smoked goat meat. Agriculture and Forestry, 68 (1): 115-130. doi:10.17707/AgricultForest.68.1.06

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DETERMINATION OF QUALITY PARAMETERS OF HERZEGOVINIAN DRY SMOKED GOAT MEAT

SUMMARY

Herzegovinian dry smoked goat meat is a traditional cured meat product made of the whole carcass of adult castrated bucks, dry salted and cold smoked. It has been traditionally produced in Herzegovina for centuries, especially in the wider area of the Stolac municipality. This study aimed to determine the quality parameters of Herzegovinian dry smoked goat meat. For the research, the samples were made into eight separate anatomical units (neck, sirloin, leg, loin, flank, breast, shoulder, hindshank), on which the tests were performed. Sensory, physical and chemical tests were performed on the examined samples. Also, to monitor changes in fats, its hydrolytic and oxidative changes (acid and peroxide number, TBARS value) were determined. The sensory evaluation determined that the examined samples were characterized by a "pleasant" aroma. Chemical tests revealed significant differences in the values of the examined parameters between samples from different anatomical regions. The least hydrolytic and oxidative changes were found in the breast samples which had the highest fat content. PCA analysis revealed a positive correlation between moisture content and pH value, as well as a negative correlation of these parameters with fat content. Furthermore, a significant positive correlation was found between NaCl content, ash, peroxide number, and TBARS values. Fat content was characteristic in the breast samples, moisture in the shoulder samples, protein in the hindshank samples, while NaCl and ash content were characteristic in the neck samples.

Keywords: quality, traditional product, sensory and physical attributes, Herzegovina, chemical parameters

INTRODUCTION

Dried meat products are highly valued products in the diet of the population and represent a good source of protein of great biological value.

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Traditional, preserved cured meat products that originate from a certain geographical area are characterized by specific sensory properties, and as a rule, top quality. The properties and quality of these products are significantly influenced, among other things, by the general characteristics of the climate, and especially by specific climatic conditions of a certain geographical area (Radovanović et al., 2005; Dumić, 2008). Traditionally, meat processing is a means of extending shelf-life (preserving) and producing a convenient item for later use. Processing is aimed at reducing the enzyme activity in the meat, retarding oxidation of the fat, and preventing spoilage by microorganisms. These aims have been achieved through drying, curing with salt, or smoking meat. Either one or a combination of these procedures in various regions of the world has preserved goat's meat (Casey et al., 2003).

Goat meat is an important source of proteins worldwide, especially in developing countries. It has about the same nutritional value as sheep meat (contains more proteins and less fat compared to sheep meat) (Ivanović et al., 2014). The most important component of goat muscle tissue is, of course, proteins, whose nutritional value depends on the presence of certain essential amino acids. According to the presence of amino acids, goat meat fully meets the nutritional needs of an adult person (Ivanković et al., 2013). Goat meat, due to its high protein content, low amount of intramuscular and subcutaneous fat, the richness of macro and microelements and water, and fat-soluble vitamins are of high nutritional value (Kegalj et al., 2011). Also, the amount of fat, especially cholesterol, is low (Troskot & Pavičić, 2007). The chemical composition of goat meat, and more specifically intramuscular fat, is influenced by factors such as live weight, genotype, muscle, gender, and diet (Madruga & Bressan, 2011).

In Bosnia and Herzegovina, the processing of goat meat is symbolic and is mainly reduced to the thermal processing of goat meat (goat roasting) and the production of traditionally cured meat products (goat "stelja", "pastrma", "plaha" and dry smoked goat meat). One of them is the autochthonous "Herzegovinian dry smoked goat meat". The production of this traditional product is specific to the area of Eastern Herzegovina, and especially to the wider locality of the municipality of Stolac. The peculiarity of the Mediterranean climate and plant cover, the specificity of the smell and aroma of meat, as well as the production technology, gives this traditional meat product exceptional sensory properties (Ganić et al., 2019; 2021). According to the Regulation of ground meat, meat preparations and meat products (Official Gazette of B&H No. 82/13), Herzegovinian dry smoked goat meat is classified as a preserved dried meat product where the moisture content must be less than 60%, water activity less than 0.93 and it must be stored at temperatures up to 15 ℃. In Bosnia and Herzegovina, goat meat is most often consumed in the form of roast goat meat. Very small quantities are processed exclusively as dried meat products - goat "stelja". This way of meat technological processing is characteristic of certain areas such as Central Bosnia and Eastern Herzegovina. The production of goat "stelja" has an exclusively artisanal character, and a very small number of artisanal meat processors are engaged in the production. In the last few years, based on the experience of producers, there has been a significant demand for cured meat products made from goat meat. This trend is probably a consequence of consumers being better informed about the nutritional characteristics of this type of meat. Also, meat processors point out that in the last two years (during the Covid 19 pandemic), there has been an enormous demand for goat "stelja" and tallow (goat fat).

"Herzegovinian dry smoked goat meat" is a traditional meat product, by which the area of Eastern Herzegovina, primarily the municipalities of Stolac and Berkovići, was recognizable in the past. Production was characteristic of the hill and mountain areas of these municipalities. Based on the oral traditions of the domicile population, in the middle of the 20th century, about 200 "plahas" of dried goat were sold per week at the local market in Stolac. Bearing in mind the fact that in the last 10 to 15 years, there is a strong trend of extinction and displacement, especially in hilly and mountainous rural areas, this production has been reduced to a minimum. Nowadays, in the wider locality of the municipality of Stolac, there is only one meat processor engaged in the production of "Herzegovinian dry smoked goat meat". Its annual production is around 150 "steljas", which are mainly placed on the Sarajevo market (market places). Based on the current circumstances, it is very likely that the production of this indigenous product will be shut down soon. On the other hand, the tradition, quality, popularity of the product, and the ubiquitous trend of consuming indigenous food, impose the need to preserve this product, i.e. its production. For this purpose, pioneering research on the technology and quality of "Herzegovinian dry smoked goat meat" was conducted. The obtained results will be used in the process of protection of this product with the label of authenticity on the national level. With the protection of "Herzegovinian dry smoked goat meat", the product would gain even more popularity. Preconditions would be created for its more significant production, and in the future, it could certainly have exceptional export potential. Local communities would certainly invest more in the infrastructure of rural areas, which would give the local population an incentive to stay on their properties and have a secure source of income. In addition to the above, the mentioned localities have exceptional potential for the development of rural tourism. In this regard, the production of Herzegovinian dry smoked goat meat would fit perfectly into such projects.

Available literature data indicate modest scientific work on the processing and quality of goat meat in Bosnia and Herzegovina. Ganić et al., (2013; 2019; 2020) and Forto (2020) cite significant scientific research on the technology and quality of cured meat products from goat meat. In contrast to the above mentioned, in other countries, the quality of various goat meat products is continuously tested, such as smoked goat ham (Ivanović et al., 2014; 2014a; 2016), goat mantas (Oliveira et al., 2014), goat bresaola (Paleari et al., 2003), salted and ripened goat thigh (Paleari et al., 2008), goat cured legs (Teixeira et al., 2017; Tolentino et al., 2016).

The research aims to record the production technology for the first time and to establish the basic quality parameters of "Herzegovinian dry smoked goat meat".

MATERIAL AND METHODS

Production technology of Herzegovinian dry smoked goat meat. To produce "Herzegovinian dry smoked goat meat", only castrated bucks over three years of age are used. The meat processor, which produces this meat product, owns its property on Hrgud mountain, where the animals stay all year round. Bucks intended for the production of "Herzegovinian dry smoked goat meat" are grazed exclusively on pasture, without the addition of a concentrated meal. The production of this traditional meat product is characteristic only for the period from December to February. For this research, five adult castrated bucks, older than three years, were selected.

Standard technology involves the following phases: bucks selection, slaughter, head and skin separation, evisceration, carcass cooling, deboning and processing of raw “stelja”, salting, then drying and smoking. Bleeding of animals is done traditionally. The neck veins at the level of the atlanto-occipital joint are cut with a knife. After the bleeding is over, decapitation (separation) of the head and skin is performed. The boning and processing of the trunk begin by making an incision in the part of the sternum, towards the spinal column and the neck. The meat is then separated from the bones by dissecting the musculature and the pelvic symphysis (*Symphisis pelvis*). In the next phase, an incision is made on the cranial side of the hind limbs with separate musculature of the femoral (*Regio femoris*) and crural regions (*Regio cruris*). Eventually, the skeleton is completely separated from the musculature. The shoulders remain within the trunk, with the shoulder blade (*Scapula*) and the upper arm bone (*Humerus*) being deboned, while the forearm bone (*Radius*) remains within the muscle tissue. To remove the shoulder blade and the upper arm bone, an incision is made on the medial side and the capsule articularis is opened, whereby *cavitas glenoidalis* (cavity) and *caput humeri* (head) are observed.

The obtained raw “plaha” is additionally processed and shaped, whereby the salt is removed from the leg part so that the added salt penetrates better into the meat, and so that the smoking done is as complete as possible. When salting, only coarse crystalline sea salt is used, which is sprinkled on the meat (dry salting). Salting lasts only a few days (three to four days). After that, timber is introduced into the smokehouses for drying and smoking. Also, this part of the process varies according to weather conditions and lasts from a minimum of 15 to 20 days, depending on the temperature and airflow. It is desirable to smoke intermittently every two days for 24 hours. Apart from smoking and drying, all technological phases were performed in the slaughterhouse "Obradović" Stolac. Smoking and drying of meat are done in traditional stone smokehouses on the mountain Hrgud (above 1000 m).

Figure 1. Production technology of Herzegovinian dry smoked goat meat (a - deboning of thoracic part; b - deboning of axial skeleton without head, pelvis, and femur; c - salting; d - smoking)

Methods

Sampling. Research in this paper has been conducted in the period from January to June 2019.

For the research, five “steljas” were used (Fig. 2). Eight samples (neck, sirloin, leg, loin, flank, breast, shoulder, hindshank) from different anatomical parts were taken from each “stelja” (Fig. 3). The shoulder pattern in Fig. 3 is not visible because it is located on the lateral side of the “stelja”.

Figure 2. Herzegovinian dry smoked goat meat

Figure 3. Sampling of Herzegovinian dry smoked goat meat

Chemical analysis. Moisture content was determined by BAS ISO 1442:2007, protein content by BAS ISO 937:2007, fat content by BAS ISO 1443:2007, ash content by ISO 936:2007, pH was measured using pH meter (FiveGo^{TM} F2, Mettler Toledo, Switzerland), and a_w value of the samples was measured using an a_w meter (LabSwift – a_w, Novasina, Switzerland). Determination of NaCl was performed by Mohr titration using 0.1 M $AgNO_3$ and 5% potassium chromate as an indicator (E. K. 8045, JUS., 1993).

Indicators of lipolysis and lipid oxidation. Acid value (AV) was used as an indicator of lipolysis. The acid value was determined according to BAS EN ISO 660:2010 method and expressed as mg KOH/g fat. The level of lipid oxidation was assessed by the determination of peroxide value (primary oxidation) and by the Thiobarbituric acid assay (secondary oxidation). Peroxide value (PV) was determined according to BAS EN ISO 3960:2018 method and expressed as mmol/kg. Thiobarbituric acid (TBA) assay was conducted according to Lemon (1975). The method is based on the spectrophotometric determination of the pink color that results from the reaction between Thiobarbituric acid (TBA) and malondialdehyde (MDA - ketoaldehyde, which is a secondary product of lipid oxidation of unsaturated fatty acids). Spectrophotometric absorbance readings were performed at 538 nm. A spectrophotometer (Perkin Elmer Lambda 25 UV/VIS, 190-1100 nm) was used to read the absorbances. The concentration of pigment formed was determined by reading from a standard curve where the TBARS values obtained were expressed as mg malondialdehyde/kg sample (mg MDA/kg sample). A calibration curve was developed using 0, 0.01, 0.02, 0.03, 0.04 and 0.05 μmol of malondialdehyde (MDA).

Sensory analysis. Sensory analysis was performed by a simple descriptive method (Radovanović & Popov-Raljić, 2000). When describing sensory properties of Herzegovinian dry smoked goat meat, the most frequent and appropriate descriptive terms were used (Stamenković & Dević, 2006). The sensory evaluation was performed by a three-member expert commission that performs evaluations for fairs and exhibitions. No standard scoring was applied in this study. This methodology of the sensory evaluation was used to identify for the first time the sensory and physical characteristics of individual segments of "Herzegovinian dry smoked goat meat". The facts will serve as a basis and corrective for future research on this product. Measurements of the dimensions of individual tissues were performed using a movable scale with an accuracy of 0.00 mm. Samples were cut into slices using a professional meat slicer (Meat Slicer AWT FS-190-15A) and served on plastic plates 30 minutes before the start of the sensory evaluation. For performing the sensory evaluation, the ambient temperature up to 20 °C and the presence of daylight is provided. About 50 mL of water and 15 g of unsalted bread were provided to assessors between successive samples.

Statistical analyses. All determinations (chemical parameters, indicators of lipolysis and lipid oxidation) were carried out in triplicate, and data were reported as mean ± standard deviation. Influence of anatomical regions was tested using one-way ANOVA, and significant differences ($p<0.05$) were calculated using Tukey test. For the correlation and presentation of the results multivariate data analysis was used - analysis of the basic components or PCA analysis. Statistical analyses were performed using Past software 3.15 (Hammer *et al.*, 2001).

RESULTS AND DISCUSSION

Chemical analysis. Results of the chemical analysis are shown in Table 1. Through statistical processing of the obtained results, it was established that the anatomical region had a statistically significant influence on all chemical parameters. Moisture content was in the range of 12,61-24,94%. The highest moisture content was in the hindshank samples (24,94%), while the lowest content was in the breast samples (12,61%). Higher moisture content compared to those determined in this paper are stated by Ganić *et al*., (2013) in goat "stelja", Ivanović *et al*., (2014; 2014a; 2016) in smoked goat ham, Paleari *et al*., (2003) in goat bresaola, Paleari *et al*., (2008) in salted and ripened goat thigh, Oliveira *et al*., (2014) in goat mantas, Teixeira *et al*., (2017) in goat cured legs, Ganić *et al*., (2009; 2013a) and Dumić (2008) in sheep ham, Gajić (2000) and Krvavica *et al*., (2009) in sheep kastradina. All tested samples comply with the provisions of the Regulation of ground meat, meat preparations, and meat products (Official Gazette of B&H No. 82/13) in terms of moisture content because the recorded values were less than 60%.

Table 1. Chemical parameters of Herzegovinian dry smoked goat meat

Part of carcass	**Parameters (n=5)**						
	Moisture (%)	**Fat (%)**	**Protein (%)**	**NaCl (%)**	**Ash (%)**	**pH**	**a_w**
Neck	$18,49^{c}$ ± 0,16	$33,71^{f}$ ± 0,30	$26,39^{b}$ ± 0,66	$9,99^{f}$ ± 0,14	$11,41^{f}$ ± 0,23	$6,14^{d}$ ± 0,04	$0,721^{abc}$ ± 0,00
Sirloin	$17,32^{b}$ ± 0,56	$39,99^{g}$ ± 0,21	$25,79^{b}$ ± 0,79	$7,90^{d}$ ± 0,15	$9,00^{d}$ ± 0,17	$5,58^{b}$ ± 0,04	$0,716^{a}$ ± 0,00
Leg	$24,02^{e}$ ± 0,24	$24,58^{c}$ ± 0,31	$38,94^{d}$ ± 0,46	$5,55^{a}$ ± 0,01	$6,91^{a}$ ± 0,22	$6,05^{cd}$ ± 0,03	$0,754^{d}$ ± 0,00
Loin	$24,44^{e}$ ± 0,06	$26,16^{d}$ ± 0,21	$30,99^{c}$ ± 0,35	$8,50^{e}$ ± 0,07	$9,91^{e}$ ± 0,11	$6,00^{bcd}$ ± 0,06	$0,728^{c}$ ± 0,00
Flank	$21,02^{d}$ ± 0,23	$30,14^{e}$ ± 0,42	$32,73^{c}$ ± 0,19	$7,38^{c}$ ± 0,13	$8,73^{cd}$ ± 0,18	$6,17^{d}$ ± 0,08	$0,720^{ab}$ ± 0,00
Breast	$12,61^{a}$ ± 0,23	$50,35^{h}$ ± 0,15	$23,81^{a}$ ± 0,57	$5,79^{a}$ ± 0,18	$7,43^{ab}$ ± 0,23	$5,56^{a}$ ± 0,07	$0,722^{abc}$ ± 0,00
Shoulder	$24,68^{e}$ ± 0,25	$23,10^{b}$ ± 0,35	$37,53^{d}$ ± 0,45	$6,52^{b}$ ± 0,07	$8,17^{c}$ ± 0,00	$5,89^{bc}$ ± 0,06	$0,725^{bc}$ ± 0,00
Hindshank	$24,94^{e}$ ± 0,23	$14,40^{a}$ ± 0,17	$47,40^{e}$ ± 0,36	$5,69^{a}$ ± 0,06	$7,57^{b}$ ± 0,09	$6,06^{cd}$ ± 0,02	$0,727^{bc}$ ± 0,00

Different letters within the same column indicate statistically significant differences ($p<0.05$)

The fat content was determined in the range of 14,40-50,35%. The hindshank samples had the lowest fat content (14,40%). In contrast, the breast samples had the highest fat content (50,35%) which had the lowest moisture content. These results are consistent with the results reported by Ganić *et al*., (2013; 2009; 2013a), Gajić (2000), and Krvavica *et al*., (2009). The fat content determined by Ivanović *et al*., (2014; 2014a; 2016) in smoked goat ham is in the range determined in this paper. In the examined samples, the protein content ranged from 23,81% (breast) to 47,40% (hindshank).

The protein content determined in the samples of Herzegovinian dry smoked goat meat is following the results recorded by Ivanović *et al*., (2014; 2014a; 2016), Paleari *et al*., (2003; 2008), and Oliveira *et al*., (2014). The neck samples had the highest content of NaCl (9,99%) and ash (11,41%). On the other hand, the samples of the leg had the lowest content of the mentioned components (5,55%; 6,91%). The lower content of these components was determined by Ganić *et al*., (2009; 2013), Ivanović *et al*., (2014; 2014a; 2016), Paleari *et al*., (2008) in their research.

Samples of Herzegovinian dry smoked goat meat had a pH value of 5,56-6,17. The stated values are under the established values for smoked goat ham (Ivanović *et al*., 2014; 2016), goat cured legs (Teixeira *et al*., 2017; Tolentino *et al*., 2016), goat mantas (Oliveira *et al*., 2014) and salted and ripened goat thigh (Paleari *et al*., 2008). Samples of Herzegovinian dry smoked goat meat had an a_w value of 0.716-0.754 and showed the values typically associated with intermediate moisture foods, which include dry-cured meat products (Leistner, 1991). Higher values of the examined parameter were found for smoked goat ham (Ivanović *et al*., 2014a), goat bresaola (Paleari *et al*., 2008), goat mantas (Oliveira *et al*., 2014), goat cured legs (Teixeira *et al*., 2017; Tolentino *et al*., 2016). All tested samples in terms of a_w values were under the Regulation of ground meat, meat preparations, and meat products (Official Gazette of B&H No. 82/13) because the values of this parameter did not exceed 0,93.

Indicators of lipolysis and lipid oxidation. The acid value, peroxide value, and TBARS value of Herzegovinian dry smoked goat meat are presented in Table 2. Through statistical processing of the obtained results, it was established that the anatomical region had a statistically significant influence on the stated parameters. The acid number of the tested samples ranged from 9,24 to 20,69 mg KOH/kg fat. The highest value of the acid number was found in the samples of the shoulder, which did not have the highest fat content. The activity of lipolytic enzymes increases with the increase of the moisture content, which is probably due to higher values of the acid number in the shoulder samples, which was characterized by higher moisture content. Also, the content of n-3 fatty acids increases the sensitivity to lipolytic changes, which may be another reason for these results. The lowest value of the acid number was found in the breast samples that had the highest fat content. The reason for these results is probably the higher content of more stable saturated fatty acids, which cannot be explained without a detailed analysis of the fatty acid composition. These results are in line with the results reported by Krvavica *et al*., (2016) for kastradina, and Kurćubić and Vesković-Moračanin (2017) for beef prosciutto. Higher values of the acid number were found by Krvavica *et al*., (2013; 2021) for Istrian ham, in their research.

The neck samples had the highest values of peroxide value (4,65 mmol/kg) compared to other tested samples.

Table 2. Indicators of lipolysis and lipid oxidation of Herzegovinian dry smoked goat meat

Part of carcass	Parameters (n=5)		
	Acid value (AV), mg KOH/g fat	**Peroxide value (PV), mmol/kg**	**TBARS, mg MDA/kg sample**
Neck	9,60[a] ± 0,13	4,65[f] ± 0,28	1,02[f] ± 0,01
Sirloin	17,74[d] ± 0,22	2,35[bcd] ± 0,18	0,57[d] ± 0,00
Leg	19,51[e] ± 0,18	1,87[ab] ± 0,19	0,78[e] ± 0,01
Loin	14,68[b] ± 0,40	2,82[d] ± 0,13	1,08[g] ± 0,01
Flank	20,48[f] ± 0,13	2,60[cd] ± 0,18	1,17[h] ± 0,00
Breast	9,24[a] ± 0,15	1,53[a] ± 0,04	0,47[b] ± 0,00
Shoulder	20,69[f] ±0,26	3,78[e] ± 0,15	0,50[c] ± 0,00
Hindshank	16,50[c] ± 0,20	2,03[abc] ± 0,13	0,28[a] ± 0,01

Different letters within the same column indicate statistically significant differences (p<0.05)

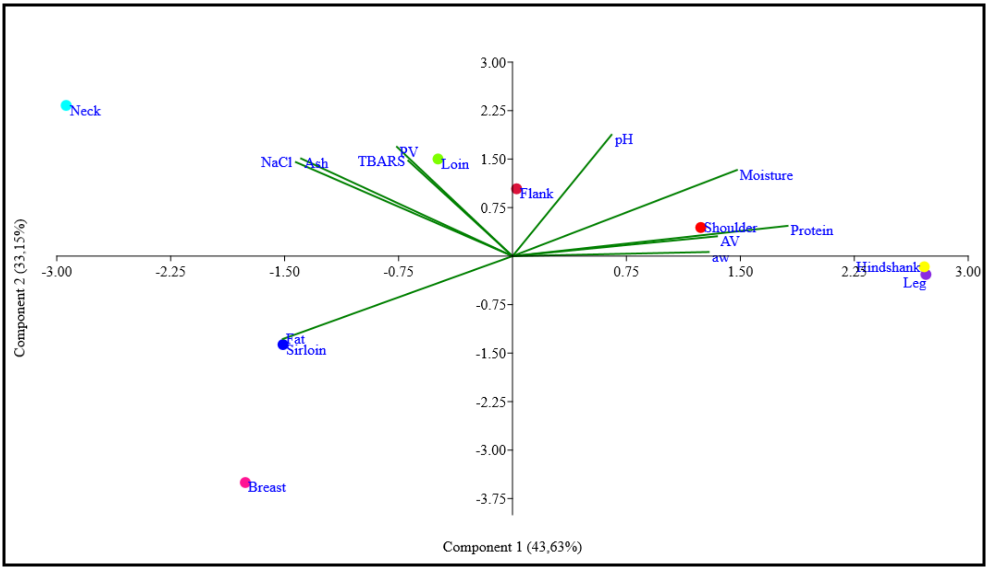

Figure 4. The plot of principal component analysis of the chemical parameters and indicators of lipolysis and lipid oxidation of Herzegovinian dry smoked goat meat

These samples had the highest NaCl content (9,99%) which is probably another promoter of lipolysis. Higher content of linoleic fatty acid and PUFA intensify oxidative changes, while higher content of SFA "protects" samples from oxidation. The breast samples had the lowest peroxide value (1,53 mmol/kg) although they had the highest fat content which is probably due to the higher content of more stable SFA. Higher values of the peroxide number were found by Krvavica *et al*., (2016) for kastradina and Krvavica *et al*., (2013; 2021) for Istrian ham.

The TBARS value ranged from 0,28-1,17 mg MDA/kg sample in the tested samples. The hindshank samples had the lowest TBARS value, while the flank samples had the highest value. Higher TBARS values cite Oliveira *et al*., (2014) for goat mantas. These results are by the values given for Istrian ham by Marušić *et al*., (2011; 2014) and Krvavica *et al*., (2021), for dalmatian dry-cured pork (Krvavica *et al*., 2016a) and dalmatian ham (Marušić Radovčić *et al*., 2016).

Sensory analysis. The results of the sensory evaluation of Herzegovinian dry smoked goat meat are shown in Table 3.

Table 3. Sensory and physical attributes of Herzegovinian dry smoked goat meat

Neck	The external appearance of this anatomical part of the trunk indicates a dominant presence of muscle tissue, permeated with thin layers of golden-yellow adipose tissue. The presence of adipose tissue is especially noticeable on the inner (medial) part of the neck, where the muscle surface is almost completely covered in a thin layer of adipose tissue of lighter shades of yellow. The cross-section is dominated by muscle tissue. When chewing, the flesh of the neck is hard. The fragility in the mouth is very weak due to the presence of parts of the connective tissue (tendon). The smell of the meat is reminiscent of smoke, without the pronounced salinity and the characteristic pungent smell of goat meat. And due to the pronounced dryness, it is harder to cut into thin slices.
Sirloin	On the outer part of the sirloin are mutually differentiated surfaces of muscle and parts with predominantly adipose tissue. The muscular layer is dark brown (chocolate) shade, while the surface with the dominant adipose tissue is golden-yellow. In contrast, the inner sirloin region is divided into the central muscle part and the adipose tissue areas located at the periphery of the sirloin. The color of the muscle tissue is distinctly dark chocolate shade, while the adipose tissue with darker tones is yellow. In cross-section, the muscle tissue is dominant about adipose tissue in a ratio of 2:1. In the area of the sirloin, next to the back muscle, fat is more present, while in the part next to the abdomen, muscle tissue dominates. The structure is compact without the noticeable presence of cracks. Due to the dryness, it is difficult to cut into thin steaks. Muscle tissue is crumbly when chewed, and adipose tissue does not lag the palate when crushed in the mouth. The presence of salt is optimally expressed, and the smell and aroma are reminiscent of smoke and have a slightly pronounced aroma.

Leg	Due to insufficient drying and smoking, the meat in the cross-section is distinctly red (the color of fresh meat). Marbling is present but to a much lesser extent than in loin samples. The layer of adipose tissue is thin and covers only the outer part of the leg (the part on which the skin rests). The flesh has a fine unique structure throughout the cross-section and no visible cracks. When minced in the mouth, the meat is juicy and soft. The crumbs in the mouth are very good and the fat does not lag on the palate. The smell is less pronounced and slightly reminiscent of smoke. The average “stelja“ thickness on the leg part was 40,60 mm (middle part of the area of M. *gastrocnemius*), 21,80 mm on the section of M. *glutaeobiceps* and M. *semitendinosus*, and 35,00 mm of the area of M. *extensor digitorum longus* (Neil, 1964).
Loin	The color of the muscle tissue on the outer part of the loin is brown, while the adipose tissue is golden-yellow. It is dominated by muscle tissue. In the inner part (medial) of the loin, adipose tissue is more present, which has a significantly lighter tone compared to the lateral part. The cross-section is dark brown (chocolate) shades, with the dominant presence of muscle tissue. At the cross-section, a monolithic structure without cracks is noticeable, with a very small presence of adipose tissue. Compared to the neck and sirloin, it is much easier to cut into 2 mm thick slices. When chewing, adipose tissue sticks to the palate. It has no pronounced salinity. The smell is reminiscent of smoke, without the exaggerated aroma of dry goat meat.
Flank	This anatomical part of the trunk is made up of almost equally muscle and adipose tissue. The color of the muscle parts is a closed red shade, while the adipose tissue is yellow. It is relatively well cut into 2 mm thick slices. It has good crumbliness when chewed. The aroma has a present aroma of smoked goat meat, with a pungent taste and aroma.
Breast	Differentiated adipose and muscle layer, dominated by the adipose tissue part. The color of the muscle tissue on the inside is dark red with brown tones, while the color of the adipose tissue is white to light yellow. The meat is easily cut into 2 mm thick slices, it is crumbly in the mouth and the fat does not stick to the palate. The smell of the “stelja“ is characteristic and reminiscent of smoke. The taste of primarily adipose tissue is strong, astringent, and unpleasant. The thickness of the samples was 19,30 mm in the middle part, 19,10 mm towards *Linea alba* and the area of *regio xyphoide*, and in the part towards the ribs, the thickness was 17,70 mm.
Shoulder	The outer part of the muscle tissue is dark red to brown shades. The surfaces of adipose tissue are golden-yellow to brown. The smell is reminiscent of smoked meat, without the presence of overemphasized aroma of goat meat. It can be relatively easily cut into 2 mm thick slices. It has good crumbliness when chewed in the mouth. Muscle and, to a lesser extent, adipose tissue is present in this anatomical part of the trunk.

Hindshank	The extremely dry muscle tissue of dark brown color. Very difficult to cut into 2 mm thick slices. Not overly pronounced smell of smoked goat meat. When chewed in the mouth, it shows exceptional hardness. At this anatomical position of the trunk, muscle tissue is dominant.

Relationship between chemical parameters and indicators of lipolysis and lipid oxidation. The analysis of the main components was performed based on a correlation matrix in which 10 parameters were included for eight samples of Herzegovinian dry smoked goat meat. For the analysis of the main components, a_w and pH value, moisture, fat, ash, protein, NaCl content, acid number, peroxide number, TBARS value were used as variables. The first two components that are the result of testing the above parameters of samples of Herzegovinian dry smoked goat meat contained 76,78% of the total variance, the first 43,63%, and the second 33,15%. The cumulative variance for the four main components was 93,53%.

From the results shown in Fig. 4, the moisture content and pH value achieved a significant positive correlation, while the stated parameters were in a significant negative correlation with the fat content. A positive correlation was found between the content of NaCl, ash, peroxide, and TBARS value. Also, a high positive correlation was found between protein content, a_w value, and acid number. From the presented graph the fat content was characteristic for the breast samples. The hindshank samples had a characteristic protein content, while the shoulder samples had characteristic moisture content. In contrast, the content of NaCl and ash was characteristic of the neck samples. TBARS value was characteristic of the flank samples, a_w value of the leg samples, peroxide value of the neck samples, and an acid value of the shoulder samples.

CONCLUSIONS

Herzegovina dried goat meat is a valuable food product thanks to its specific sensory properties and favorable chemical composition. From the aspect of sensory quality, this product is characterized by a pleasant aroma, smell of smoke, and slightly pronounced salinity, except for samples of the flank and breast. The samples of the flank had a pronounced smell of smoked goat meat, with a pungent taste and aroma, and this anatomical part of the carcass is characterized by almost equally muscular and fatty tissue. The breast samples had an adipose tissue taste that was strongly pronounced, astringent, and unpleasant due to the dominance of adipose tissue in this anatomical part, and which usually contained the compounds responsible for taste and aroma. The analysis of basic chemical indicators showed significant differences between individual anatomical parts. The most intense hydrolytic changes were found in the shoulder samples in which the acid value was 20,69 mg KOH/g fat. The highest value of the peroxide number was found in the neck samples (4,65 mmol/kg), and TBARS value in the flank samples (1,17 mg MDA/kg).

The results of the research will be used to standardize the production and quality of "Herzegovinian dry smoked goat meat". The determining technological and qualitative parameters will be used for the manufacturer's product specification in the process of its protection with the label of authenticity. With the protection of "Herzegovinian dry smoked goat meat" on the national level, the product will be officially branded and will gain even more in popularization and marketing. With certain technological additions (confectioning into smaller pieces, vacuum packaging, and labeling), it can represent an exceptional development opportunity for the rural areas where it is produced. Having in mind the above, there is an urgent need to protect this meat product, unique in Bosnia and Herzegovina and beyond.

REFERENCES

BAS EN ISO (2010): Animal and vegetable fats and oils - Determination of acid value and acidity No. 660. The Institute for standardization BiH.

BAS EN ISO (2018): Animal and vegetable fats and oils - Determination of peroxide value - Iodometric (visual) endpoint determination No. 3960. The Institute for standardization BiH.

BAS ISO (2007): Meat and meat products - Determination of moisture content (Reference method) No. 1442. The Institute for standardization BiH.

BAS ISO (2007): Meat and meat products - Determination of nitrogen content (Reference method) No. 937. The Institute for standardization BiH.

BAS ISO (2007): Meat and meat products - Determination of total ash No. 936. The Institute for standardization BiH.

BAS ISO (2007): Meat and meat products - Determination of total fat content No. 1443. The Institute for standardization BiH.

Casey, N. H., Van Niekerk, W. A. & Webb, E. C. (2003): Goat meat. In: Caballero, B., Trugo, L., Finglass, P. (Eds.), Encyclopaedia of Food Sciences and Nutrition. Academic Press, London, pp. 2937–2944.

Dumić, S. (2008): Ispitivanje važnijih svojstava kvaliteta sjeničke stelje kao osnova za zaštitu oznake porekla. Magistarski rad. Poljoprivredni fakultet Univerziteta u Beogradu. Srbija.

E. K. 8045, JUS (1993): Određivanje sadržaja natrijum-hlorida. Službeni list SRJ 27/93.

Forto, A. (2020): Kvalitativna svojstva „Hercegovačke suhe jarčetine“. Master rad. Poljoprivredno-prehrambeni fakultet Sarajevo. BiH.

Gajić, B. (2000): Kontaminiranost suhomesnatih proizvoda supstancama štetnim po zdravlje ljudi. Magistarski rad. Poljoprivredno–prehrambeni fakultet Univerziteta u Sarajevu. BiH.

Ganić, A., Čaušević, A., Karahmet, E., Stojković, S. & Ratković, D. (2013a): Contribution of technology and quality ham of sheep. International 57th Meat Industry Conference: Meat and meat products - perspectives of sustainable production, Belgrade, Serbia. Proceedings, pp. 215-220.

Ganić, A., Forto, A. & Begić, M. (2019): Qualitative parameters of Herzegovina's dry-smoked goat for the protection of its at national level. 12th International Scientific and Professional Conference WITH FOOD TO HEALTH. Osijek, Croatia, Book of Abstracts, pp. 152.

Ganić, A., Karahmet, E., Čaklovica, F. & Zahirović, L. (2013): The quality of goat "stelja" dependenton anatomic position. *MESO*, XV (1): 50-57.

Ganić, A., Krvavica, M., Begić, M. & Forto, A. (2021): Volatile flavour compounds of Herzegovina's dry-cured goat. 10th Central European Congress on Food (CEFood) Sarajevo. Book of Abstracts, pp. 80.

Ganić, A., Smajić, A., Bijeljac, S., Brdarić, N., Zahirović, L., Jesenković, L., Operta, S. & Omanović, H. (2009): Comparasion of basic quality parameters of sheep prosciutto produced in handicraft and industrial conditions. XX Scientific and Expert Agriculture and Food Industry Conference Neum. Proceedings, pp. 117-123.

Hammer, Ø., Harper, D. A. T. & Ryan, P. D. (2001): PAST: Paleontological Statistics software package for education and data analysis. *Palaeontologia Electronica* 4 (1): 1-9.

Ivanković, M., Ivanković, S., Ćavar, S., Šubara, M., Batinić, V., Čurković, M., Pocrnja, D., Puljić, L., Bijeljac, S., Boras, A. & Lasić, M. (2013): Kozarstvo. Agronomski i prehrambeno-tehnološki fakultet Sveučilišta u Mostaru. BiH.

Ivanović, S., Baltić, M., Nesić, K., Pisinov, B., Pavlović, I., Ivanović, J., Živković, D. & Marković, R. (2014a): Hygiene of smoked goat ham production. *Journal of Pure and Applied Microbiology*, 8 (3): 2155-2162.

Ivanović, S., Nešić, K., Pisinov, B. & Pavlović, I. (2016): The impact of diet on the quality of fresh meat and smoked ham in goat. *Small Ruminant Research*, 138: 53–59.

Ivanović, S., Pisinov, B., Bošković, M., Ivanović, J., Marković, R., Baltić, M. & Nesić, K. (2014): Changes in the quality of goat meat in the production of smoked ham. *Tehnologija mesa*, 55 (2): 148–155.

Kegalj, A., Mioč, B. & Vrdoljak, M. (2011): Kakvoća kozjeg mesa. *Stočarstvo*, 65 (1): 55-65.

Krvavica, M., Friganović, E., Đugum, J. & Kegalj, A. (2009): Dalmatian kastradina (kostradina). *MESO*, XI (5): 285-290.

Krvavica, M., Gugić, J., Friganović, E., Tomić, N., Konjačić, M. & Uremović, M. (2013): The effect of genotype on chemical properties of Istrian dry-cured ham. *MESO*, XV (1): 38-43.

Krvavica, M., Jelić, M., Velić, A., Lučin, M. & Gajdoš Kljusurić, J. (2016a): The physical characteristics and oxidative status of dalmatian dry-cured pork loin manufactured in different technological conditions. *MESO*, XVIII (5): 421-421.

Krvavica, M., Konjačić, M. & Mioč, B. (2016): Effect of sex, castration and various technological processes on chemical composition of sheep meat and kastradina. *MESO*, XVIII (3): 228-237.

Krvavica, M., Lasić, D., Kljusurić, J. G., Đugum, J., Janović, Š., Milovac, S. & Bošnir, J. (2021): Chemical characteristics of Croatian traditional Istarski pršut (PDO) produced from two different pig genotypes. *Molecules*, 26 (14), 4140: 1-17.

Kurćubić, V. & Vesković-Moračanin, S. (2017): Comparative testing of quality of beef prosciutto produced in a traditional and industrial manner. XXII Biotechnology counselling. Proceedings, Book 2, pp. 585-590.

Leistner, L. (1991): Fermented and intermediate moisture meat products. *Outlook on Agriculture*, 20: 113−119.

Lemon, D. W. (1975): An improved TBA test for rancidity. New series. Circular No. 51. Halifax, Nova Scotia, Halifax Laboratory.

Madruga, M. S. & Bressan, M. C. (2011): Goat meats: Description, rational use, certification, processing and technological developments. *Small Ruminant Research*, 98 (1-3): 39–45.

Marušić Radovčić, N., Vidaček, S., Janči, T. & Medić, H. (2016): Characterization of volatile compounds, physico-chemical and sensory characteristics of smoked dry-cured ham. *Journal of Food Science and Technology*, 53 (11): 4093–4105.

Marušić, N., Petrović, M., Vidaček, S., Petrak, T. & Medić, H. (2011): Characterization of traditional Istrian dry-cured ham by means of physical and chemical analyses and volatile compounds. *Meat Science*, 88 (4): 786–790.

Marušić, N., Vidaček, S., Janči, T., Petrak, T. & Medić, H. (2014): Determination of volatile compounds and quality parameters of traditional Istrian dry-cured ham. *Meat Science*, 96 (4): 1409–1416.

Neil, D. S. May (1964): The anatomy of the sheep. University of Queensland Press, St. Lucia, Brisbane, Queensland.

Oliveira, A. F., Rodrigues, S., Leite, A., Paulos, K., Pereira, E. & Teixeira, A. (2014): Short Communication: Quality of ewe and goat meat cured product *mantas*. An approach to provide value added to culled animals. *Canadian Journal of Animal Science*, 94 (3): 459–462.

Paleari, M. A., Moretti, V. M., Beretta, G. & Caprino, F. (2008): Chemical parameters, fatty acids and volatile compounds of salted and ripened goat thigh. *Small Ruminant Research*, 74 (1-3): 140–148.

Paleari, M. A., Moretti, V. M., Beretta, G., Mentasti, T. & Bersani, C. (2003): Cured products from different animal species. *Meat Science*, 63: 485–489.

Pravilnik o usitnjenom mesu, poluproizvodima i proizvodima od mesa (Službeni glasnik BiH br. 82/13).

Radovanović, R. & Popov-Raljić, J. (2000): Senzorna analiza prehrambenih proizvoda. Poljoprivredni fakultet Beograd - Tehnološki fakultet Novi Sad.

Radovanović, R., Tomić, N., Tomašević, I. & Rajković, A. (2005): Prinos muskulature namijenjene proizvodnji „Goveđe užičke pršute“. *Tehnologija mesa*, 46 (5–6): 250–264.

Stamenković, T. & Dević, B. (2006): Sensory properties of the sheep prosciutto. *Tehnologija mesa* 47, (3-4): 115-122.

Teixeira, A., Fernandes, A., Pereira, E., Manuel, A. & Rodrigues, S. (2017): Effect of salting and ripening on the physicochemical and sensory quality of goat and sheep cured legs. *Meat Science*, 134: 163-169.

Tolentino, G. S., Estevinho, L. M., Pascoal, A., Rodrigues, S. S. & Teixeira, A. J. (2016): Microbiological quality and sensory evaluation of new cured products obtained from sheep and goat meat. *Animal Production Science*, 57: 391-400.

Troskot, A. & Pavičić, Ž. (2007): Proizvodnja i kakvoća kozjeg mesa. *MESO*, IX (1): 43-46.

Xhulaj, Bode, D., Koto, R. (2022): Estimation of genetic variability of autochthonous wheat (Triticum aestivum L.) genotypes using multivariate analysis. Agriculture and Forestry, 68 (1): 131-143. doi:10.17707/AgricultForest.68.1.07

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ESTIMATION OF GENETIC VARIABILITY OF AUTOCHTHONOUS WHEAT (*TRITICUM AESTIVUM* L.) GENOTYPES USING MULTIVARIATE ANALYSIS

SUMMARY

This study was conduct to evaluate the morphological variability of 23 autochthonous wheat genotypes (*Triticum aestivum* L.) part of the base collection of the Gene Bank (Agricultural University of Tirana). Crop production is strongly related with genetic diversity within germplasm and its improvement. Various statistical techniques have been used to study diversity among different genotypes. Among these techniques multivariate is most frequently used one for the genetic association of genotypes. Principal components and cluster analysis were carried out involving quantitative traits, such as tiller capacity, plant height, spike length, number of spikelet per spike, number of seeds per spikelet, seed size, weight of seeds per spike and seed yield. According to PCA, three components exhibited about 67.15% of the variability within 23 wheat genotypes. Accessions were grouped into three major clusters based on Euclidean distance, suggesting a variance of 41.72% within classes and 58.28% between classes. Accessions with major level of dissimilarity between them were AGB 3071 (Univers 6) and AGB 3064 (IKBA_05). The results suggested that plant height, spike length, number of spikelet per spike and weight of seeds per spike were the most important characters in differentiating the genotypes. The use of principal component and correlation coefficient analysis in the wheat germplasm, simplify dependable classification of genotypes, the identification of the superior genotypes and their relation with morphological traits with possibility expenditure in breeding programs.

Keywords: cluster, PC, genotypes, morphological, traits, variability

INTRODUCTION

Agriculture in Albania is still a significant sector of the economy which contributes to 22.5% of the country's GDP providing the income base for most of the population and serves as an employment safety net. Wheat and especially

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bread wheat (*Triticum aestivum* L.) is one of the most important cultivated food crops in Albania, with 70 000 ha of cultivated area.

The priority of wheat improving program is to increase grain yield. Any improvement program for increasing yield requires sufficient information on their parental materials in respect of variation for yield and yield contributing characters.

The evaluation of genetic variability based on morphological characters especially those of economic interest might be used to select appropriate materials in breeding programs for crop improvement (Dos Santos *et al.* 2009). Various statistical techniques have been used to study diversity among different genotypes. Among these techniques multivariate is most frequently used one for the genetic association of genotypes (Ali *et al.* 2021).

Quantitative traits are often used to assess and describe the wheat characters due to their role in the estimation of genetic diversity and discrimination of closely related types (Al Khanjari *et al.* 2008). The correlation coefficient analysis is useful in the identification of characters that are positively correlated with yield (Maqbool *et al*. 2010). This analysis is a reliable statistical method, which provides tool to quantify the associations among different traits and indicate whether the influence is reflected in the yield (Sabaghina *et al*. 2014).

The Institute of Plant Genetic Resources (former Albanian Gene Bank) has a total of 270 accessions of bread wheat genotypes (*Triticum aestivum* L.) in long-term storage (https://www.eurisco.ipk-gatersleben.de/apex/). This gene bank has the aim not only to preserve the germplasm but also to make available the plant resources into breeding programs, to improve cultivars or to develop new ones.

Therefore, the characterization of plant germplasm (accessions) maintained in active or base collections in such institutions, and the examination of the genetic relationship between them, is an important tool for the sustainable conservation and certainly to increase use of crop genetic resources. This study seeks to be attached to previous studies conducted by this institution (Bode *et al*. 2012; 2013; 2015; Cakalli *et al.* 2017; Xhulaj *et al.* 2018), on the evaluation and characterization of different plant genetic resources held in conservation, in order to enrich the database (passport data) with additional biological information, essential for sustainable collection management but also for direct use in agriculture.

The object of this study was the assessment of diversity, the evaluation of possible interrelationships among yield contributing traits through characterization of 23 accessions of bread wheat (*Triticum aestivum* L.), will help in selecting the desirable characters and serves as a valuable source for creation of new gene combinations to sustain common wheat future breeding programs.

MATERIAL AND METHODS

The study is based on the characterization of the diversity of bread wheat (*Triticum aestivum* L) germplasm. The plant material is characterized by a survey on land and laboratory, in the experimental field of the Agricultural University of Tirana.

Plant Material: Object of the study, 23 genotypes of bread wheat (*Triticum aestivum* L.), part of the base collection (Table 1, https://www.eurisco.ipk-gatersleben.de/apex/).

Experimental site: The study was conducted at the Experimental Station of Institute of Plant Genetic Resources Valias, Tiranë (altitude of 40 m above sea level and at Latitude 41°24'6.14"N and Longitude 19°44'9.93"E).

Table 1. List of the 23 wheat (*Triticum aestivum* L.) accessions object of the study

Accession code	Accession name	Origin	Acquire date
AGB 0330	-	Albania	2003/01/08
AGB 0331	-	Albania	2003/01/08
AGB 0332	-	Albania	2003/01/08
AGB 0333	-	Albania	2003/01/08
AGB 0334	-	Albania	2003/01/08
AGB 0335	-	Albania	2003/01/08
AGB 0336	-	Albania	2003/01/08
AGB 0337	-	Albania	2003/01/08
AGB 0338	-	Albania	2003/01/08
AGB 0339	-	Albania	2003/01/08
AGB 0340	-	Albania	2003/01/08
AGB 3066	Univers 1	Albania	2010/11/25
AGB 3067	Univers 2	Albania	2010/11/25
AGB 3068	Univers 3	Albania	2010/11/25
AGB 3069	Univers 4	Albania	2010/11/25
AGB 3070	Univers 5	Albania	2010/11/25
AGB 3071	Univers 6	Albania	2010/11/25
AGB 2837	Golia	Albania	2001/06/26
AGB 2838	Agimi	Albania	2001/06/26
AGB 2840		Albania	2001/06/26
AGB 2842		Albania	2001/06/26
AGB 3064	IKBA_05	Albania	2010/11/25
AGB 3065	IKBA_06	Albania	2010/11/25

Methods (Experimental Design): Experiment carried two replication during the autumn season 2017-2018. During the crop year, the accessions were evaluated for different characters of quantitative type as: tiller capacity (TC), plant height/cm (PH), spike length/cm (SL), number of spikelet per spike (NSpkSp), number of seeds per spikelet (NSSpk), number of seeds per spike (NSSp), weight of seeds per spike/g (WSSp), weight of 1000 seeds (W1000S) and seed yield/g (SY). The wheat accessions were also evaluated for qualitative traits as: awnedness, glumes color, glumes hairiness, seed color and seed virtuousness. Morphological characterization of the accessions was conducted according to international standards (IPGRI, 1985).

Statistical analyses: Statistical tests were carried out by the Statistical Package for Social Sciences (version 21).

RESULTS AND DISCUSSION

The genetic variability among 23 autochthonous wheat genotypes stored in the Gene Bank is assessed using different agronomic traits, especially those related to yield component as plant height, spike length, number of spikes per spike and grain weight. Basic statistic for qualitative traits (Table 2) and quantitative traits (Table 3) were used and the estimated variation coefficient was high for agronomic traits as number of seeds per spike, spike length, weight of seeds per spike and weight of 1000 seeds, similar with others authors (Ali *et al*. 2008; Sabaghina *et al*. 2014). Regarding qualitative traits 47.82% of the bread wheat genotypes presented conspicuous awns (awned), while 26.08% short awns and AGB 3069, AGB 2837, AGB 2838, AGB 3064 and AGB 3065 with no awns. Major part of wheat genotypes 65.21% after transversely section appear partly vitreous while 34.78% of wheat accessions non vitreous (Table 2).

Table 2. Data of qualitative morphological traits of 23 *Triticum aestivum* L. genotypes

Accession code AGB	Awnedness	Glumes color	Glumes hairiness	Seed color	Seed Size	Vitreousness
0330	awned	red to brown	absent	purple	large	partly vitreous
0331	awned	red to brown	absent	red	large	partly vitreous
0332	awned	red to brown	low	red	intermediate	partly vitreous
0333	awned	red to brown	low	red	large	partly vitreous
0334	awned	red to brown	absent	red	large	partly vitreous
0335	awned	white	low	red	large	partly vitreous
0336	awned	white	absent	white	large	partly vitreous
0337	awned	purple to black	high	red	large	non vitreous
0338	awned	red to brown	absent	red	small	partly vitreous
0339	awned	white	absent	red	small	partly vitreous
0340	awned	red to brown	high	red	large	partly vitreous
3066	awn less	red to brown	low	purple	small	non vitreous
3067	awnletted	red to brown	low	purple	small	partly vitreous
3068	awnletted	red to brown	absent	red	intermediate	partly vitreous
3069	awn less	red to brown	absent	red	small	non vitreous
3070	awnletted	red to brown	absent	white	small	partly vitreous
3071	awnletted	red to brown	high	red	intermediate	partly vitreous
2837	awn less	red to brown	absent	red	small	non vitreous
2838	awn less	red to brown	absent	white	small	non vitreous
2840	awnletted	red to brown	low	purple	large	partly vitreous
2842	awnletted	red to brown	low	purple	intermediate	non vitreous
3064	awn less	white	absent	purple	intermediate	non vitreous
3065	awn less	purple to black	low	purple	intermediate	non vitreous

Variability is observed for seed size trait, 34.78% of the genotypes presented small size, 26.08% intermediate size and 39.13% of the wheat genotypes presented large seed size. Wheat grain yield is the result of some important physiological traits occurring in the growth and mostly is determined by the number of spikes, the number of grains and grain weight (Zecevic *et al*. 2010; Sabaghina *et al*. 2014). Results for tiller capacity trait reveal two accessions AGB 0332 and AGB 0334 with the highest value (2.6) while 9 from 23 wheat genotypes presented the same lowest value. AGB 3068 recorded the lowest value for four quantitative traits as tiller capacity, plant height, spike length and number of seeds per spikelet while AGB 2840 the highest value for two traits as, plant height and spike length (14, 72 cm). Results presented for spike length are higher from those reported from other authors (Peltonen *et al*. 2007; Xhulaj *et al.* 2017). Genotype AGB 2838 presented the highest values for number of seeds per spikelet and number of seeds per spike. As previously reported (Sabaghina *et al.* 2014; Xhulaj *et al.* 2020) grain yield is influenced by spike properties and the spikelet number plays a very important role in the wheat grain yield. AGB 0338 recorded the highest value (25.8) and the lowest one at AGB 3064 (16.2).

Table 3. Descriptive statistics of quantitative traits in 23 genotypes of bread wheat (*Triticum aestivum* L.)

Code AGB	TC	PH	SL	NSpkSp	NSSpk	WSSp	W1000S	NSSp
0330	2.3±0.2	93.5±1.7	8.4±0.2	23.8±0.3	3.0±0.1	1.92±0.1	40.0±0.1	54.0±0.5
0331	2.5±0.2	91.7±0.6	8.1±0.1	20.2±0.3	3.0±0.1	2.4±0.1	48.0±0.1	59.8±1.2
0332	2.6±0.1	91.7±0.9	8.4±0.1	19.4±0.4	3.0±0.1	2.66±0.2	50.1±0.5	62.0±1.9
0333	2.0±0.1	97.6±0.7	8.2±0.1	20.8±0.2	3.0±0.1	2.42±0.1	51.0±0.1	48.0±2.3
0334	2.6±0.1	99.8±0.9	9.1±0.2	21.6±0.4	3.0±0.1	2.38±0.1	41.7±0.1	57.0±0.5
0335	2.0±0.1	101.8±0.6	8.3±0.1	23.0±0.1	3.0±0.1	2.62±0.1	57.0±0.1	46.0±0.4
0336	2.5±0.2	99.8±1.4	7.8±0.1	23.6±0.3	3.0±0.2	1.64±0.1	39.6±0.5	41.6±1.0
0337	2.0±0.3	97.5±0.8	8.2±0.2	21.6±0.3	3.0±0.1	2.34±0.5	50.1±0.7	56.0±1.3
0338	2.3±0.2	105.1±1.3	9.4±0.2	25.8±0.3	3.0±0.1	1.44±0.2	39.2±0.1	54.8±1.2
0339	2.2±0.2	99.3±1.2	8.2±0.1	24.6±0.3	3.0±0.2	2.1±0.3	54±0.1	52.8±1.4
0340	2.4±0.2	96.6±1.3	7.8±0.2	21.6±0.3	3.0±0.1	2.74±0.2	50.2±0.3	58.9±1.4
3066	2.0±0.1	101.7±0.6	13.7±0.1	23.4±0.3	2.0±0.1	1.42±0.2	30.1±0.1	38.0±0.8
3067	2.0±0.7	73.5±1.1	11.3±0.2	22.6±0.4	3.0±0.2	1.44±0.1	35.2±0.5	50.0±1.8
3068	2.0±0.1	71.7±0.6	7.1±0.1	20.4±0.3	2.0±0.2	1.96±0.2	34±0.7	55.8±0.1
3069	2.0±0.5	93.5±1.0	9.5±0.1	19.8±0.3	2.0±0.1	1.46±0.1	31.1±0.1	18.6±0.2
3070	2.2±0.2	83.3±0.8	10.9±0.2	22.6±0.5	3.0±0.1	1.84±0.2	41.3±0.7	56.4±1.2
3071	2.0±0.2	73.5±0.8	12.2±0.1	23.2±0.5	3.0±0.1	1.88±0.1	39.1±0.7	69.0±1.5
2837	2.0±0.3	72.5±0.8	8.5±0.2	21.4±0.3	3.0±0.2	2.26±0.2	34±0.5	57.8±1.1
2838	2.4±0.2	88.0±0.8	11.4±0.2	22.2±0.3	4.0±0.4	2.72±0.2	39±1.2	72.4±0.4
2840	2.0±0.8	136.2±2.1	14.7±0.5	18.2±0.3	3.0±0.1	2.0±0.1	45±1.5	48.4±1.2
2842	2.1±0.1	129.3±1.5	12.2±0.4	19.8±0.3	3.0±0.1	2.32±0.3	42.3±0.3	28.4±0.2
3064	2.4±0.2	102.3±0.8	10.2±0.2	16.2±0.3	4.0±0.2	2.61±0.3	47±1.2	66.4±1.8
3065	2.5±0.2	83.5±1.1	10.1±0.2	19.4±0.6	3.0±0.2	2.1±0.2	32±1.3	65.6±1.5
Min.	2.01	71.70	7.10	16.20	2.00	1.42	30.10	18.60
Max.	2.60	136.2	14.72	25.80	4.00	2.74	57.00	72.40
Mean	2.21	94.93	9.73	21.53	2.95	2.11	42.21	52.94
St.deviation	0.22	15.49	1.99	2.151	0.46	0.42	7.482	12.25
CV	0.10	0.16	0.20	0.10	0.15	0.20	0.17	0.23

Three wheat genotypes presented the lowest values measured for number of seeds per spikelet trait (AGB 3066, AGB 3068 and AGB 3069) while two other accessions are similar for the same trait with 4 numbers of seeds per spikelet (AGB 2838 and AGB 3064). High variation is observed between wheat germplasm for weight of seeds per spike from 1.42 at AGB 3066 to 2.74 (AGB 0340). Seed weight parameter also is important in wheat increasing seed germination percent, seedling emergence, tiller capacity, spike density and yield (Bellatreche *et al.* 2017). AGB 3070 recorded the highest value for weight of 1000 seeds (57g). According to Okamoto *et al.* (2013) the grain number and weight as two main components in wheat grain yield are determined at different times of the growing season. This author suggested that seed weight best-explained genotype by environmental interaction for wheat grain yield.

Regarding plant height trait was observed a high variation among 23 wheat germplasm, whereas AGB 3068 recorded the lowest value (71.1cm) and AGB 2840 the highest value (136.2cm). The results are higher from those reported by other authors (Sabaghina *et al.* 2014) for the same trait (54.9 cm to 109.53 cm), whereas Mahmood *et al.* (2006) obtained results ranging from 62 cm to 110 cm, while Aliu *et al.* (2010) reported a range from 71 to 79 cm in different bread wheat genotypes.

Correlation Coefficient Analysis:

To measure the interdependence between a pair of characters, correlation of quantitative morphological traits was calculated by studying the data of 23 bread wheat germplasm (Table 4).

Table 4. Correlation matrix among the morphological traits (Pearson (n))

Variables	TC	PH	SL	NSpkSp	NSSpk	WSSp	W1000S	NSSp	SY
TC	1	0.019	-0.295	-0.105	0.429	0.354	0.139	0.425	0.339
PH	0.019	1	0.368	-0.208	0.111	0.110	0.357	-0.429	0.060
SL	-0.295	0.368	1	-0.144	0.043	-0.304	-0.343	-0.130	-0.293
NSpkSp	-0.105	-0.208	-0.144	1	-0.160	-0.422	-0.047	-0.034	-0.053
NSSpk	0.429	0.111	0.043	-0.160	1	0.573	0.413	0.607	0.319
WSSp	0.354	0.110	-0.304	-0.422	0.573	1	0.656	0.462	0.250
W1000S	0.139	0.357	-0.343	-0.047	0.413	0.656	1	0.180	0.247
NSSp	0.425	-0.429	-0.130	-0.034	0.607	0.462	0.180	1	0.241
SY	0.339	0.060	-0.293	-0.053	0.319	0.250	0.247	0.241	1

Knowledge of correlation is required to obtain the expected response of other traits when selection is applied to the trait of interest in a breeding program (Maqbool *et al.* 2010). Tiller capacity trait exhibited positive significant correlation with number of seeds per spikelet (r=0.429), number of seeds per spike (r=0.425) and weight of seeds per spike (r=0.354). It presented negative relations with spike length and number of spikelets per spike.

Positive relationship is observed between plant height and traits as spike length (r= 0.368) and weight of 1000 seeds (r=0.357) (Zecevic *et al*. 2004; Bilgin *et al*. 2011). Strong negative correlation is observed among plant height and number of seeds per spike (r=-0.429). Spike length exhibited negative correlation with most of the traits especially with weight of 1000 seeds (r=-0.368). Number of spikelet per spike it is negatively correlated with all of the traits.

At genotypic level number of seed per spikelet possessed positive significant correlation with yield contributing traits as the number of seeds per spike (r=0.607) and weight of seed per spikelet (r=0.573). Strong positive correlation is observed among weight of 1000 seeds and weight of seeds per spike (r=0.656). This trait exhibited negative correlation with spike length (r=-0.343). Number of seeds (grains) per spike is positive related with weight of seeds per spike (r=0.462) but strongly negative related to plant height. Positive relationship is observed among seed yield and two other traits as tiller capacity (r=0.339) and number of seeds per spikelet (r=0.319). Similar results for positive correlations between yield components such as tiller capacity and number of seeds per spikelet's with grain yield are reported from different authors (Desheva *et al*. 2016; Okuyama *et al.* 2004; Zecevic *et al*. 2004; Xhulaj *et al.* 2019).

Principal Component analysis:

Results of PCA suggest that 77.50% of the variability is exhibited among 23 common wheat genotypes by four components (Table 5) where the two first components influenced mostly the variability (F1 34.54% and F2 18.89% Figure 1).

Table 5. Eigen values and % total variance for PCA in 23 accessions of wheat

PCA	F1	F2	F3	F4	F5	F6	F7	F8	F9
Eigen value	3.109	1.700	1.235	0.932	0.884	0.633	0.242	0.165	0.099
Variability (%)	34.54	18.88	13.72	10.35	9.826	7.033	2.694	1.838	1.104
Cumulative %	34.54	53.43	67.15	77.50	87.33	94.36	97.05	98.89	100.00
Eigenvectors									
TC	0.359	-0.137	0.098	0.346	-0.360	0.708	-0.128	-0.023	-0.274
PH	0.022	0.683	-0.185	0.332	0.015	0.193	-0.130	0.012	0.578
SL	-0.222	0.401	0.578	0.259	0.214	-0.135	-0.304	0.144	-0.460
NSpkSp	-0.156	-0.380	-0.310	0.469	0.604	0.132	-0.050	0.362	0.007
NSSpk	0.440	0.096	0.315	0.213	0.305	-0.057	0.738	-0.103	0.027
WSSp	0.479	0.162	-0.034	-0.390	0.047	0.003	-0.091	0.762	-0.034
W1000S	0.370	0.248	-0.454	-0.142	0.389	-0.025	-0.228	-0.448	-0.414
NSSp	0.382	-0.329	0.417	-0.029	0.237	-0.107	-0.507	-0.221	0.445
SY	0.303	-0.072	-0.214	0.514	-0.396	-0.641	-0.098	0.089	-0.090

The variability presence within the first component was related with number of seed per spikelet and weight of seeds per spike traits at the level of 42.26% but poor in plant height. This last trait strongly contributes at the level of 46.61% of the genotypes variability in the second component, but was very poor in number of seeds per spikelet (0.92%). The third principal component exhibited positive effects for spike length trait (33.44%) and number of seeds per spike (17.4%). The seed yield trait was responsible for the variability at the fourth principal component at the level of 26.38%. According to this analyze, the variables that effect most the variability within the forth components are plant height and number of seeds per spikelet.

In addition to cluster analysis, the biplot (genotype by trait) has been applied to study the relation among studied traits in a set of genotypes (Aghaee *et al*. 2010; Peterson *et al.* 2005).

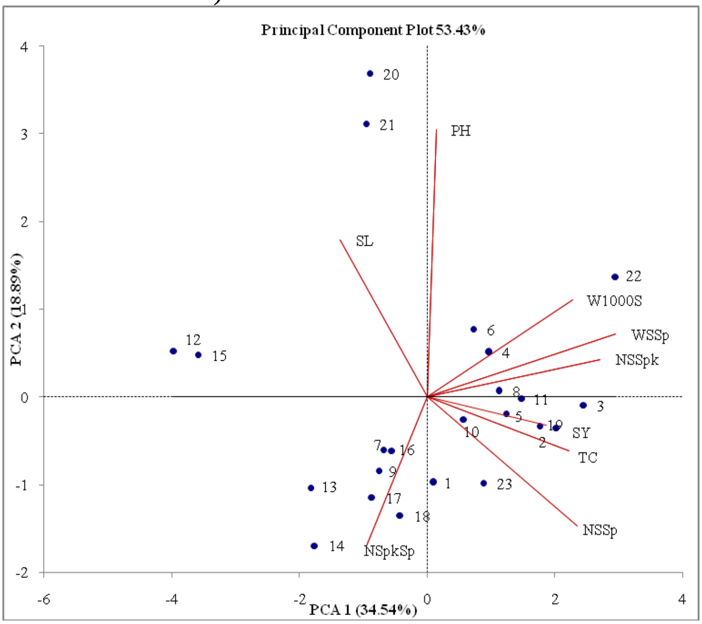

Figure 1. Principal component biplot of 23 wheat (*Triticum aestivum* L.) accessions

The biplot (Figure 1) suggest that the best or the incompatible wheat genotypes in most of the traits, since they had the longest distance from the origin for the two principal components were AGB 2840 (20), AGB 2842 (21), AGB 3064 (22), AGB 0332 (3) and AGB 2838 (19). Therefore it seems that for the first PC wheat genotypes (numbered at Figure 1) AGB 3064 (22), AGB 2838 (19) and AGB 0340 (11) have the highest values mostly for number of seeds per spikelet's, number of seeds per spike and weight of seeds per spike traits, while genotypes as AGB 0332 (3) and AGB 0334 (5), resulted with the highest values basically for tiller capacity trait.

The genotypes that presented not suitable performance for the measured traits within the first component, with lower distance from the origin of the biplot were AGB 3066 (12), AGB 3069 (15), and AGB 3067 (13). According to the PC analyze the wheat genotypes that presented the highest variability for the traits in the second component were AGB 2840 (20), AGB 2842 (21), AGB 0335 (6) and AGB 0333 (4) basically for plant height and spike length, and the other related cultivars which fall in its sector were suitable for the same traits too. Within this component the group of genotypes AGB 3068 (14), AGB 3071 (17), AGB 2837 (18) and AGB 3065 (23) resulted with the lowest performance for the measured traits (Figure 1).

The vector view of the biplot suggest a strong positive correlation among traits, as indicated by the small obtuse angles between their vectors, especially between tiller capacity of the genotypes and seed yield, number of seeds per spike. Also this positive relation (r=cos 0= +1) is observed through weight of seeds per spike and thousand-seed weight and number of seeds per spikelet. The correlation between traits, plant height and tiller capacity, weight of seeds per spike, seed yield and finally number of seeds per spikelet's, was near zero (r=cos 90= 0) as indicated by the near perpendicular vectors. The existence of a strong negative correlation of the vectors, indicated by the near angle of approximately 180 degrees is observed between NSSP and PH, SL; also among WSSP and NSpkSp. Comparing the Eigen values for each factor using the minimum Eigen value criterion, there are 3 main PC with Eigen values > 1.00 (Table 5 and Figure 1) that influence the genetic variability among 23 wheat genotypes.

The first principal component showed 34.54 % of variability with Eigen value 3.109 in germplasm which then reduced gradually. After the fourth component little variance was observed and it ended at 1.1 % (Eigen value 0.09). From the graph (Figure 1) the maximum variation was present in the first component so the selection of genotypes with desirable traits from this principal component will be useful for further breeding programs.

Cluster analysis:

Genetic diversity among autochthons wheat germplasm was calculated after Agglomerative hierarchical clustering (AHC, Wards method). The tree (Figure 2.) was divided in three major clusters. The first cluster include 11 wheat accessions with AGB 0337 as central observation, basically grouped for similarity in traits as tiller capacity, number of seeds per spikelet, weight of 1000 seeds and plant height but different in number of seeds per spike and weight of seeds per spike. Two most closely wheat genotypes within this cluster are AGB 0337 (observation 8) and AGB 0340 (observation 11 with 3.13 Euclidean distance), fully similar for traits as number of spikelet per spike, number of seeds per spikelet. These two accessions were joined from another sub-cluster formed by AGB 0331 (observation 2) and AGB 0332 (observation 3). The second cluster grouped five accessions with its central observation AGB 3066. These wheat genotypes presented similarity in traits as tiller capacity, weight of seeds per spike and weight of 1000 seeds, but were different in plant height, spike length and number of seeds per spike.

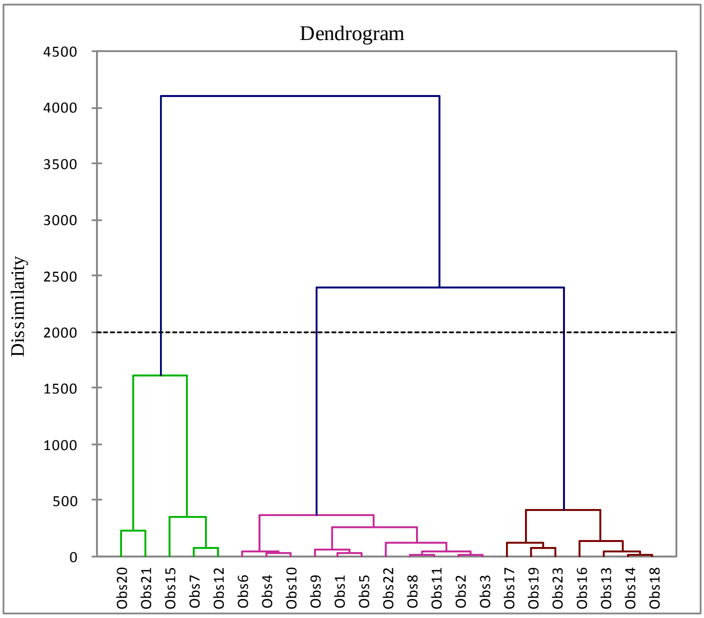

Figure 2. Dendrogram from cluster analysis of 23 bread wheat accessions based on quantitative traits

The third cluster included 7 wheat germplasm that presented similarity for the major part of the quantitative traits but very different for number of seeds per spike. Sub cluster at this group are AGB 2837 (observation 18) and AGB 3068 (observation 14) with the lowest dissimilarity level presented not only in this cluster but among the 23 wheat genotypes. These accessions were mostly similar for traits as tiller capacity, plant weight and weight of 1000 seeds. High dissimilarity level is observed among wheat genotypes part of the second cluster and the third one. The two most different wheat genotypes, with the highest dissimilarity level measured between them are AGB 2842 (observation 21) and AGB 3071 (observation 17). Genetic dissimilarity values of these two accessions basically consists for traits as plant height, number of spikelet per spike, weight of seeds per spike, weight of 1000 seeds and number of seeds per spike. Other pairs of wheat genotypes with high dissimilarity level between them are AGB 2842 (observation 20) with AGB 3071 (observation 17) and with observation 14 (AGB 3068), basically for traits as plant height, spike length, number of spikelet per spike, weight of 1000 seeds and number of seeds per spike. These traits are also responsible for the level of dissimilarity that exist between observation 21 (AGB 2842) of the second cluster and observation 18 (AGB 2837) of the third cluster. The results reveal that the genetic distance that exist between these wheat genotypes is based in yield contributing characters as tiller capacity, weight of 1000 seeds and number of seeds per spike. According to Singh *et al*. (2018) the knowledge of genetic variability for yield contributing components helps in the improvement of grain yield and planning of effective breeding program.

CONCLUSIONS

Results of this study succeed in obtaining important scientific information on autochthonous wheat germplasm stored in the Institute of Plant Genetic Resources, and for further wheat breeding programs. The significant differences found in the present study show the existence of a high genetic variability among the 23 bread wheat genotypes and quantitative traits analyzed, adequate for selection of desirable traits, and creation of new favorable gene combinations. Results for tiller capacity trait reveal two accessions AGB 0332 and AGB 0334 with the highest value. AGB 3068 recorded the lowest value for four quantitave traits as tiller capacity, plant height, spike length and number of seeds per spikelet while AGB 2840 the highest value for two traits plant height and spike length. AGB 2838 presented the highest values for number of seeds per spikelet and number of seeds per spike.

The two most different wheat genotypes, with the highest dissimilarity level measured between them are AGB 2842 (observation 21) and AGB 3071 (observation 17). Genetic dissimilarity values of these two accessions basically consists for traits as plant height, number of spikelet per spike, weight of seeds per spike, weight of 1000 seeds and number of seeds per spike. The results reveal that the genetic distance that exist between these wheat genotypes is based in yield contributing characters as plant height, tiller capacity, thousand-seeds weight and number of seeds per spike.

At genotypic level number of seed per spikelet possessed positive significant correlation with yield contributing traits as the number of seeds per spike (r=0.607) and weight of seed per spikelet (r=0.573). Plant height showed positive significant correlation with traits as spike length (r=0.368) weight of 1000 seeds (r=0.357). Three principal components exhibited about 67.15% of variability where two PCs components influenced mostly the variability (PC1 with 34.54 % and PC2 with 18.88 %). The results suggested that plant height, spike length, number of spikelet per spike were the most important characters in differentiating the genotypes. The use of principal component analysis (showing the largest contributor to the total variance) and correlation coefficient analysis in the wheat germplasm, simplify dependable classification of genotypes, the identification of the superior genotypes (considering the evaluation of mean values) and their relation with morphological traits with possibility expenditure in breeding programs. The traits with more significant weighting on respective PC variance can be utilized successfully as quantitative markers for evaluation, characterization of the wheat germplasm stored in the gene bank.

This paper has been a contribution to increase the knowledge about the wheat germplasm collection maintained in conservation at the Gene Bank (Agriculture University of Tirana). This better understanding should allow a better conservation and use of the collection in future breeding programs. The research will also assist in the conservation of valuable germplasm, as is the case of local varieties, which are widespread use by farmers throughout the entire country.

REFERENCES

Ali, N. Hussain, I. Ali, S. Khan, N. U. & Hussain, I. (2021): Multivariate analysis for various quantitative traits in wheat advanced lines. *Saudi J Biol Sci*. 28(1):347-352. doi:10.1016/j.sjbs.2020.10.011

Ali, Y. Atta, B. M. Akhter, J. Monneveux, P. & Lateef, Z. (2008): Genetic variability association and diversity studies in wheat (*Triticum aestivum* L.) germplasm. *Pakistan Journal of Botany*, 40: 2087-2097.

Aliu, S. & Fetahu, S. (2010): Determination on genetic variation for morphological traits and yield components of new winter wheat (*Triticum aestivum* L.) lines. *Notulae Scientia Biologicae*, 2: 121-124.

Al Khanjari, S. Fialatenko, A. A. Hammer, K. & Buerkert, A. (2008): Morphological spike density of Omani wheat. *Genetic Resources Crop Evolution*, 55: 1185-1195.

Aghaee, M. Mohammadi, R. & Nabovati, S. (2010): Agro-morphological characterization of durum wheat accessions using pattern analysis. *Australian Journal of Crop Sciences*, 4(7): 505-514.

Bellatreche, A. Mahdad, M. Kaouadji, Z. & Gaouar, S. (2017): Agro-morphological diversity of some accessions of bread wheat (*Triticum aestivum* L.) in western Algeria. *Biodiversitas Journal*, 18(1): 409-415.

Bilgin, O. Korkut, Z. K. Başer, İ. Dağlioğlu, O. Öztürk, İ. Kahraman, T. & Balkan, A. (2011): Genetic variation and inter-relationship of some morpho-physiological traits in durum wheat (Triticum Durum (L.) Desf.). Pak. J. Bot., 43(1): 253-260.

Bode, D. Elezi, F, Gixhari, B. Shehu, D. & Hobdari, V. (2012): Evaluation of some morphological characteistics in maize (*Zea mays* L.) *Journal Agriculture and Forestrey*, 58(2): 65-73.

Bode, D. Elezi, F. & Gixhari, B. (2013): Morphological characterisation and interrelationships among descriptors in *Phaseolus vulgaris* accessions. *Journal Agriculture and Forestrey*, 59(2):175-185.

Bode D. (2015): Exploring the diversity richness of 30 accessions of *Phaseolus vulgaris* at Albania Gene Bank. *International Journal of Green and Herbal Chemistry*. 4(2): 193-202.

Cakalli, A. Ismaili, H. Kullaj, E. Shishmani, E. & Bode D. (2017): Evaluationg the multiplication of kiwi (*A.deliciosa*) with the cuttings treated by some rooting hormones. *International Journal of Current Microbiology and Applied Sciences.* 6(3): 2128-2133.

Desheva G. (2016): Correlation and path-coefficient analysis of quantitative characters in winter bread wheat varieties. Trakia Journal of Sciences, 14(1): 24-29.

Dos Santos, T. M. Ganança, F. Slaski, J. J. & Pinheiro de Carvalho Miguel, A. A. (2009): Morphological characterization of wheat genetic resources from the Island of Madeira, Portugal. *Genetic Resources Crop Evolution*, 56: 363-375.

IPGRI (1985): *Descriptors for Wheat (Revised)*. Institute of Plant Genetic Resources, Rome, Italy.

Mahmood, Q. Lei, W. D. Qureshi, S. & Khan, M. D. (2006): Heterosis, correlation and path analysis of morphological and biochemical characters in wheat (*Triticum aestivum* L.). *Agricultural Journal*, 1: 180-185.

Maqbool, R. Sajjad, M. Khaliq, I. Rehman, A. Khan, S. A. & Khan, H. S. (2010): Morphological diversity and trait association in bread wheat (Triticum aestivum L.). *American-Eurasian Journal of Agriculture and Environment Sciences*, 8(2): 216-224.

Okamoto, Y. Nguyen, A. T. Yoshioka, M. Iehisa, J. C. M. & Takumi, S. (2013): Identification of quantitative trait loci controlling grain size and shape in the D genome of synthetic hexaploid wheat lines. *Breeding Science*, 63: 423-429.

Peltonen-Sainio, P. Kangas, A. & Salo, Y. (2007): Grain number dominates grain weight in temperate cereal yield determination: Evidence based on 30 years of multi-location trials. *Field Crop Research*, 100: 179-188.

Peterson, D. M. Wesenberg, D. M. Burrup, D. E. & Erickson, C. A. (2005): Relationships among agronomic traits and grain composition in oat genotypes grown in different environments. *Crop Sciences*, 45: 1249-1255.

Sabaghina, N. Janmohammadi, M. & Segherloo, A. (2014): Evaluation of some agro-morphological traits diversity in Iranian bread wheat genotypes. *UMCSBIO*, 19: 79-90.

SPSS, Statistics. (2016): www.ibm.com/products/spss-statistics

Xhulaj, B. D. Hobdari, V. Shehu, D. Gixhari, B. & Elezi, F. (2017): Agro morphological characterization performance of 100 common wheat (*Triticum aestivum* L.) accessions. *Albanian Journal of Agricultural Science*, 16(2): 219-227.

Xhulaj, B. D. Hobdari, V. & Elezi, F. (2018): Evaluation of morphological diversity pattern in the Albanian barley (*Hordeum vulgare* L.) base collection. *Albanian Journal of Agricultural Science*, 17: 22-29.

Xhulaj, B. D. Elezi, F. & Hobdari, V. (2019): Interrelations among traits and morphological diversity of wheat (*Triticum aestivum* L.) accessions in base collection of Plant Genetic Resources Institute, Albania. *Acta Agriculturae Slovenica*, 113(1): 163-173. 20.

Xhulaj (Bode) D. & Gixhari, B. (2020): Analysis of genetic variation in bread wheat by grain yield components. *Agriculture and Forestry*, Vol. 66 (4): 89-100, ISSN 0554-5579. DOI: 10.17707/AgricultForest.66.4.07.

Zecevic, V. Knezevic, D. & Micanovic, D. (2004): Genetic correlations and path-coefficient analysis of yield and qualitycomponents in wheat (Triticum aestivum L.). Genetika, 36(1):13-21.

Zecevic, V. Boskovic, J. Dimitrijevic, M. & Petrovic, S. (2010): Genetic and phenotypic variability of yield components in wheat (*Triticum aestivum* L.). *Bulgarian Journal of Agriculture Science*, 16: 422-428.

Quoc, L.P.T. (2022): Physicochemical characteristics and antioxidant activities of banana peels pectin extracted with microwave-assisted extraction. Agriculture and Forestry, 68 (1): 145-157. doi:10.17707/AgricultForest.68.1.08

DOI: 10.17707/AgricultForest.68.1.08

Le Pham Tan QUOC [1]*

PHYSICOCHEMICAL CHARACTERISTICS AND ANTIOXIDANT ACTIVITIES OF BANANA PEELS PECTIN EXTRACTED WITH MICROWAVE-ASSISTED EXTRACTION

SUMMARY

Pectin production from banana peels was carried out in microwave-assisted extraction (MAE). The main purpose of this study is to determine the extraction conditions with the best physicochemical characteristics and highest antioxidant activity (AA). The pectin was isolated using the MAE method with two different solvents (citric acid and tartaric acid) at 420 and 613 W for 5 and 10 min. In this study, using MAE with tartaric acid as a solvent strongly improves the yield and AA of banana peel pectin (BPP) compared to those of BPP extracted by citric acid. BPP extracted with MAE and tartaric acid at 420 W for 10 min had the highest yield (15.23±0.52%), while the best AA was obtained at 420 W for 5 min (free radical scavenging activity of 37.17±0.7%). The chemical properties of BPP including equivalent weight, degree of esterification, methoxyl content and total anhydroronic acid, depended on the extraction conditions and BPP could be classified as a low-methoxyl pectin (LMP). The present study also proved that BPP was an ideal alternative source of commercial pectin with the high purity.

Keywords: Banana, microwave, pectin, peel, waste

INTRODUCTION

Banana is considered one of the most important tropical fruits in the Vietnam market. In Vietnam, there are many types of banana that are widely cultivated everywhere, especially Pisang Awak (*Musa acuminata* × *Musa balbisiana*). It can be processed into many kinds of foods, such as chips, crackers, banana muffins, baked bananas, fried banana cakes, etc. Therefore, banana fruit peels make up a large fraction of the waste produced from banana processing. This waste also is an important issue to the processing industries and a challenge for by-product waste management (Kamble *et al.*, 2017). The proper utilization of banana peels can enhance some economic values and reduce environmental pollution (Phaiphan, 2019). In there, using banana peels to produce pectin is one

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of the best solutions because this compound can be used in many fields; for instance, pectin is an additive in food products (thickener, stabilizer and gelling agents) (Emaga *et al.*, 2008) and it is also found in cosmetic and pharmaceutical products (Rolin, 1993).

Pectin is a complex mixture of polysaccharides found in the primary cell wall and intracellular layer of plant cells, mainly in fruits (Mudgil, 2017). The gelling mechanism of pectin strongly depends on the degree of esterification (DE). Pectin is classified into high methoxy pectin (HMP, DE>50%) and low methoxy pectin (LMP, DE<50%) (Mesbahi *et al.*, 2005). Currently, there are many methods to extract pectin from fruit waste. Among them, microwave-assisted extraction (MAE) is the best choice compared to other methods due to improved pectin yield and short extraction times. Many previous studies demonstrated these advantages mentioned above. For instance, Maran *et al.* (2013), Koštálová *et al.* (2016) and Sarah *et al.* (2018) extracted pectin from orange peel, unutilized pumpkin biomass and cocoa peel. In addition, the yield and quality of pectin also depends on solvents. Therefore, the combination of MAE and various solvents can improve the pectin yield and significantly change its characteristics, which adapt to various demands in the food industry. However, until now, there have been no studies on these combinations to extract pectin from banana waste in Vietnam. Based on these reasons, we conducted experiments to isolate pectin from banana peels using MAE, with citric acid and tartaric acid as solvents.

MATERIAL AND METHODS

Materials

Bananas originated from Tien Giang province (Vietnam) and were similar in the uniformity of shape, size and ripeness. Bananas were free of physical damage, and fungal infection. The banana peels were collected from a fried banana store in Ho Chi Minh City and removed from the pulp at the stage 5 of ripeness (more yellow than green). The pectin extraction efficiency obtained the highest level in this stage (Emaga *et al.*, 2008). In addition, all other chemicals used were of analytical reagent grade.

Sample preparation

The sample preparation was performed according to procedure of Khamsucharit *et al.* (2018) with minor modifications. Briefly, banana peels were dried under the sun until their moisture was less than 8%. Next, raw materials were ground into fine powder in a grinder (0.6 mm particle size). Then, the samples were packed in a polyethylene bag and stored at room temperature prior to analysis.

Pectin extraction process

The extraction process was based on the procedure described by Quoc *et al.* (2015) with small changes. Pectin from banana peels (5 g) was extracted using a microwave apparatus (Sanyo EM-S2182W, China) combined with citric acid (0.1 M) and tartaric acid (0.1 M), the extraction conditions were a solid-to-liquid ratio of 1:20 (g/mL), the microwave powers of 420 and 613 W for 5 and 10 min. Next, the extract was cooled and filtered using filter paper (20-25 µm pore size) to remove the residue. Then, alcohol (96%, v/v) and the obtained filtrate were mixed gently together (filtrate-to-alcohol ratio of 1:3, v/v) and the mixture was left for approximately 20 h to completely precipitate the pectin. The pectin was then filtered through cloth and purified with an alcohol (70%, v/v) solution. Pectin product was dried at 60°C in a hot air oven until its weight stabilized. Finally, it was packed and stored for further experiments.

Determination of pectin content

The percentage of pectin yield (Y) was estimated following the formula below:

$$Y = \frac{m_1}{m_0} \times 100\%$$

m_1: Weight of the obtained pectin (g); m_0: Weight of the initial sample (g).

Determination of moisture of BPP

To determine the moisture content, BPP (0.5 g) was dried in a moisture analyzer at 105°C until a constant weight was achieved. Then, the weight of the sample was recorded. The percentage of moisture content was calculated based on the moisture lost.

Determination of color parameters of BPP

The color parameters of BPP were expressed by L^*, a^* and b^* values and they were measured using a colorimeter (CS-10, China).

Determination of equivalent weight (EW) of BPP

The EW of BPP was determined according to the method reported by Owens *et al.* (1952) with minor modifications. Pectin (0.5 g) was placed in a 250 mL conical flask and wetted with 5 mL of ethanol. 100 mL distilled water and a few drops of phenolphthalein as indicator were added. The solution was titrated with 0.1 N NaOH until the color of the indicator changed to pink and kept for at least 30 s. The neutralized solution was used to determine the methoxyl content. The EW was calculated following the formula below:

$$EW\ (g/mol) = \frac{weight\ of\ sample \times 1000}{mL\ of\ alkali \times Normality\ of\ alkali}$$

Determination of the methoxyl content (MeO) and total content of anhydrouronic acid (AUA) of BPP.

The MeO and AUA were determined following the procedure of Nguyen and Pirak (2019) with slight corrections. 25 mL of 0.1 N NaOH was added into the obtained mixture from the EW analysis. Then, the mixture was shaken continuously and kept for 30 min at room temperature. Next, 25 mL of 0.1 N HCl was added into the mixture, which was also titrated by a 0.1 N NaOH solution until the color changed to pink, as before, with phenolphthalein as an indicator. This can be calculated by using the following formula:

$$MeO(\%) = \frac{normality\ of\ NaOH \times mL\ of\ alkali \times 31 \times 100}{weight\ of\ sample \times 1000}$$

$$AUA\ (\%) = \frac{176 \times 0.1 \times 100}{weight\ of\ sample \times 1000} \times (x + y)$$

where 31 is the molecular weight of the methoxyl group; x and y are the volumes (mL) of the NaOH solutions from the EW determination and MeO determination, respectively.

Determination of degree of esterification (DE) of BPP

According to Owens *et al.* (1952), the DE was estimated by the following formula:

$$DE\ (\%) = \frac{176 \times \%MeO}{31 \times \%AUA} \times 100$$

Determination of antioxidant activity (AA) of BPP

The AA of BPP was evaluated using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging method, as described by Venzon *et al.* (2015) with minor changes. A DPPH solution (0.1 mM) was dissolved in ethanol. Next, 1.5 mL of 0.1 g/mL of sample solution was mixed with 1.5 mL of DPPH solution. The obtained solution was kept for 30 min in the dark and then its absorbance was measured at 517 nm. The AA was calculated as the reduction of absorbance of the solution (%).

Fourier transform infrared spectroscopy (FTIR) spectra of BPP

The FTIR spectra of BPP were recorded on a Bruker Tensor 27 (Germany) spectrophotometer in the 4 000–500 cm^{-1} region with a resolution of 1 cm^{-1}. The sample was incorporated with potassium bromide (KBr) and pressed into a pellet prior to analysis according to the procedure of Jiang *et al.* (2012).

Scanning electron micrograph

The micromorphology of the samples and residues after the extraction process were evaluated by a scanning electron microscope (SEM) (Jeol JSM-6400, Japan). Samples were examined at 5 kV and the vacuum pressure was 0.04 Pa with various magnifications.

Data analysis

All experiments were performed in triplicate except for FTIR spectral analysis, which was performed one time. The data was analyzed using analysis of variance (ANOVA) by Statgraphics Centurion XV software (version 15.1.02, Statgraphics Technologies, Inc., USA), expressed as the mean value±standard deviation and compared using the Fisher’s least significant difference (LSD) procedure at a 5% confidence level.

RESULTS AND DISCUSSION

Physical properties of BPP

Yield of BPP

Table 1 illustrates that the pectin yield of banana peels isolated with MAE fluctuated from 5.28% to 15.23%. Using tartaric acid as a solvent significantly enhances the pectin yield compared to citric acid. In addition, an increase in extraction time can lead to an increase in the efficiency of extraction for both solvents, especially tartaric acid. This observation is similar to that of Sarah *et al.* (2018). The microwave can break down the parenchyma cells, enhance the penetration of the solvent and lead to the improvement of the pectin yield (Kratchanova *et al.*, 2004). The highest pectin yields in this study are from 14.74% to 15.23% at 420 W and 613 W for 10 min. These results were higher than those recorded by Girma and Worku (2016), who also isolated pectin from banana peels (pectin yield of 11.31%) using conventional extraction, or lower than those reported by Maran *et al.* (2013), who used microwave to extract pectin from orange peels (pectin yield of 19.24%). These results indicate that pectin yield strongly depends on the raw material, methods and extraction conditions.

Moisture of BPP

The moistures of BPP that were obtained were quite low, ranging from 3.39 to 3.83% for samples extracted using tartaric acid and from 4.99 to 6.69% for samples extracted using citric acid (Table 1).

Table 1. Yields, moisture and color parameters of BPP at various extraction conditions

Physical properties	Solvents	Microwave power (W)	Extraction time (min)	
			5	10
Pectin yield (%)	Citric acid	420	$5.28±0.68^{a}$	$5.86±0.02^{ab}$
		613	$5.51±0.39^{a}$	$6.31±0.21^{b}$
	Tartaric acid	420	$11.28±0.24^{c}$	$15.23±0.52^{e}$
		613	$12.38±0.62^{d}$	$14.74±0.15^{e}$
Moisture (%)	Citric acid	420	$4.99±0.6^{b}$	$5.77±0.06^{bc}$
		613	$6.69±1.31^{c}$	$5.94±0.19^{bc}$
	Tartaric acid	420	$3.39±0.9^{a}$	$3.83±0.49^{a}$
		613	$3.45±0.48^{a}$	$3.42±0.17^{a}$
L^*	Citric acid	420	$10.51±0.7^{bc}$	$6.98±1.3^{a}$
		613	$7.45±1.25^{ab}$	$12.68±3.73^{c}$
	Tartaric acid	420	$23.92±2.3^{d}$	$32.47±1.62^{e}$
		613	$25.98±1.41^{d}$	$29.72±1.65^{e}$
a^*	Citric acid	420	$3.44±1.17^{ab}$	$3.47±0.87^{abc}$
		613	$4.42±0.88^{bc}$	$2.82±0.73^{ab}$
	Tartaric acid	420	$5.17±0.22^{c}$	$2.72±0.38^{ab}$
		613	$3.87±1.96^{abc}$	$2.29±0.7^{a}$
b^*	Citric acid	420	$5.69±0.91^{a}$	$6.91±0.5^{a}$
		613	$5.11±0.9^{a}$	$5.31±1.31^{a}$
	Tartaric acid	420	$13.88±0.03^{c}$	$10.13±1.21^{b}$
		613	$15.28±2.34^{c}$	$11.3±1.35^{b}$

Data followed by the different superscript letters in the same physical property are significantly different ($p \leq 0.05$)

In general, all moistures are less than 10%, which is a safe moisture level for preserving the pectin powder to avoid the growth of microorganisms and the effect of pectinase on pectin quality (Mohamadzadeh *et al.*, 2010; Nguyen and Pirak, 2019). The moistures obtained in this study are in agreement with those from pectin of unripe banana peels (Kamble *et al.*, 2017) or white dragon fruit peels (Nguyen and Pirak, 2019), etc.

Color parameters of BPP

As seen in Table 1, the color parameters (L^*, a^* and b^*) of BPP had a significant difference (p<0.05). The BPP extracted using tartaric acid showed higher levels of lightness and yellowness than the BPP extracted with citric acid. The highly colored pectin was attributed to the presence of impurity components trapped inside the pectin during the precipitation process, such as phenolic compounds (Baississe *et al.*, 2010), water-soluble pigments (Nguyen and Pirak, 2019), etc. In general, as the extraction time increased, the lightness of pectin also increased for most of the samples. This trend can be explained by the fact that the phenolic compounds or water-soluble pigments are destroyed the longer the samples are kept at high temperatures, leading to the lightness of the samples. According to Khamsucharit *et al.* (2018), L^*, a^* and b^* of pectin from banana peels of different varieties in Thailand ranged from 77.33 to 85.3, 2.09 to 3.88 and 5.37 to 8.59, respectively. The L^* values of all samples in this study are significantly lower, whereas the b^* values of samples extracted using tartaric acid are higher than those mentioned above. Therefore, the color of pectin also depends on the nature and source of the fruits.

Chemical properties of BPP

EW of BPP

The EW of BPP at different conditions ranged from 188.77 to 200.82 g/mol for samples extracted using citric acid and from 222.56 to 229.71 g/mol for samples using tartaric acid (Table 2). Generally, the obtained results revealed that extraction conditions (solvents, microwave power and extraction time) dramatically influenced the EW of BPP. Compared to some previous studies, EWs of pectin from banana peels extracted using a conventional method and MAE were 925.01 g/mol (Girma and Worku, 2016) and from 1 500 to 2 300 g/eq (Rivadeneira *et al.*, 2020), respectively. Hence, the EWs in this study were quite low compared to those studies because the produced pectin had high partial degradation (Nguyen and Pirak, 2019). Additionally, Ramli and Asmawati (2011) also indicated that the EW values also depend on the amount of free acid in the samples.

MeO of BPP

Table 2 shows the MeO values of BPP. These values vary from 0.57 to 1.24% (for samples extracted with citric acid) and from 0.27 to 0.39% (for samples extracted with tartaric acid). In general, there was a tendency for the MeO values to increase slightly when extraction time increased and the microwave power does not significantly affect the MeO values. In addition, the obtained results are quite low compared to those recorded from other studies.

According to Aina *et al.* (2012), the MeO values range from 0.2 to 12%; they completely depend on extraction conditions and source of materials. For instance, the MeO values of pectin from mango peels, lemon peels, grapefruit peels, sweet orange peels and banana peels were 8.89% (Girma and Worku, 2016), 4.46, 5.79, 3.9% (Aina *et al.*, 2012) and 3.86-5.97% (Khamsucharit *et al.*, 2018), respectively. With the low MeO values (<7%), the pectin product can form gels with low concentrations of sugars compared to commercial pectins, which have MeO values ranging from 8 to 11% and can form high sugar gels (>65% sugar) (Castillo-Israel *et al.*, 2015).

AUA of BPP

As seen in Table 2, the AUA values of pectin are quite high, from 94.69 to 96.1% for citric acid and from 78.5 to 81.08% for tartaric acid. These results also showed that the microwave power and extraction time did not affect the AUA values. According to Food Chemical Codex (1996), the AUA values are the important factor to determine the purity of the extracted pectin and it is not lower than 65%. All obtained AUA values in this study fall within this range. This proved that all extracted pectin samples had a high purity and they can be used for food additives or pharmaceutical purposes. Compared to other reports, these results were higher than those of pectin from mango peels (70.65%) (Girma and Worku, 2016), Saba banana peels (39.68-57.32%) (Castillo-Israel *et al.*, 2015), etc.

Table 2. Some chemical properties of BPP at various extraction conditions

Chemical properties	Solvents	Microwave power (W)	Extraction time (min)	
			5	10
EW (g/mol)	Citric acid	420	194.31±1.9^{b}	200.57±3.26^{c}
		613	188.77±5.06^{a}	200.82±2.14^{c}
	Tartaric acid	420	224.55±0.58^{d}	229.71±1.21^{e}
		613	222.56±2.05^{d}	226.25±1.02^{de}
MeO (%)	Citric acid	420	0.97±0.13^{c}	1.3±0.22^{c}
		613	0.57±0.14^{b}	1.24±0.06^{c}
	Tartaric acid	420	0.27±0.09^{a}	0.33±0.16^{a}
		613	0.35±0.09^{ab}	0.39±0.09^{ab}
AuA (%)	Citric acid	420	96.1±1.61^{b}	95.16±2.64^{b}
		613	96.54±3.2^{b}	94.69±0.61^{b}
	Tartaric acid	420	79.9±0.7^{a}	78.5±0.61^{a}
		613	81.08±0.89^{a}	80.02±0.54^{a}
DE (%)	Citric acid	420	5.73±0.66^{c}	7.75±1.11^{d}
		613	3.36±0.71^{b}	7.44±0.41^{d}
	Tartaric acid	420	1.91±0.66^{a}	2.39±1.11^{ab}
		613	2.46±0.65^{ab}	2.78±0.65^{ab}

Data followed by the different superscript letters in the same chemical property are significantly different ($p \leq 0.05$)

DE of BPP

The results in Table 2 show that the DE values were quite low and ranged from 3.36 to 7.75% for samples extracted with citric acid and from 1.91 to 2.78% for samples extracted with tartaric acid. All these values are less than 50%. Therefore, the extracted pectin was classified as LMP (Mesbahi *et al.*, 2005).

This result contrasts with those of other studies, which showed that the DE of BPP was classified as HMP (Castillo-Israel *et al.*, 2015; Khamsucharit *et al.*, 2018). These differences can be explained by the different modes of extraction and source of initial materials.

Antioxidant activity (AA) of BPP

As seen in Table 3, it was observed that the AA of BPP strongly depends on extraction conditions. The highest AA values ranged from 35.52 to 37.17% at 420 W for 5 min (both citric and tartaric acid). Some previous studies demonstrated that pectin from plants had AA and its AA mechanism is similar to that of phenolic compounds by donating their electron to the oxidants (Serrano-Cruz *et al.*, 2013; Hung *et al.*, 2020). The obtained results also revealed that an increase in microwave power or irradiation time can lead to a significant changes in AA. This can be explained by the fact that the heat-sensitive bioactive compounds were easily degraded by the higher microwave power and longer irradiation time compared with the initial condition (420 W, 5 min). Furthermore, solvents also affect the AA of pectin because their pectin had a different amount of free carboxylic groups, resulting in different exchanges of electrons donated and finally leading to changes in the AA (Hung *et al.*, 2020). Considering that published studies on the AA of BPP are nonexistent, it is difficult to compare our results to any other findings. However, the AA of pectin has been reported for other materials, such as orange pomace (Venzon *et al.*, 2015), white dragon fruit peels (Nguyen and Pirak, 2019) and pomelo peels (Hung *et al.*, 2020), etc.

Table 3. AA (%) of BPP at various extraction conditions

Solvents	Microwave power (W)	Extraction time (min)	
		5	10
Citric acid	420	35.52 ± 1.97^{f}	8.40 ± 2.03^{a}
	613	24.44 ± 0.41^{d}	27.26 ± 0.20^{e}
Tartaric acid	420	37.17 ± 0.70^{f}	16.86 ± 1.77^{c}
	613	11.61 ± 1.26^{b}	18.24 ± 0.6^{c}

Data followed by the different superscript letters are significantly different ($p\leq0.05$)

FTIR analysis of BPP

FTIR spectra of BPP extracted using citric and tartaric acid as solvents are illustrated in Figure 1. The spectral features of all samples from 950 to 1 200 cm^{-1} were assigned to carbohydrates, especially sugar composition. The intense peaks corresponding to the characteristics of pectin polysaccharides were also shown in these spectral regions, which can be assigned to C=O stretching, C–O stretching, C–C stretching, C–H stretching and C–O bending (Wongkaew *et al.*, 2020). In addition, the absorptions of antisymmetric and symmetric stretching frequencies of the ionic carboxyl groups (COO^-) in all samples were from 1 600 to 1 650 cm^{-1} and from 1 400 to 1 450 cm^{-1} for both solvents (Posé *et al.*, 2012). The strong absorptions recorded at approximately 1 700 cm^{-1} determined the presence of the C=O stretching vibration of ester carbonyl groups (George *et al.*, 2015, Hung *et al.*, 2020). These findings are similar to the report of Posé *et al.* (2012). According to Coates (2000) and Hung *et al.* (2020), the intense absorptions of around 3 000 cm^{-1} for all samples showed the distension of –CH, $–CH_2$, $–CH_3$ and methyl esters of galacturonic acid, while the peaks ranging from 3 300 to 3 450 cm^{-1} indicated the distension of a –OH group. In general, some major components obtained from FTIR spectra were in agreement with those of pectin from other materials, such as orange pomace (Venzon *et al.*, 2015), pomelo peels (Hung *et al.*, 2020) and unripe banana peels (Kamble *et al.*, 2017), etc.

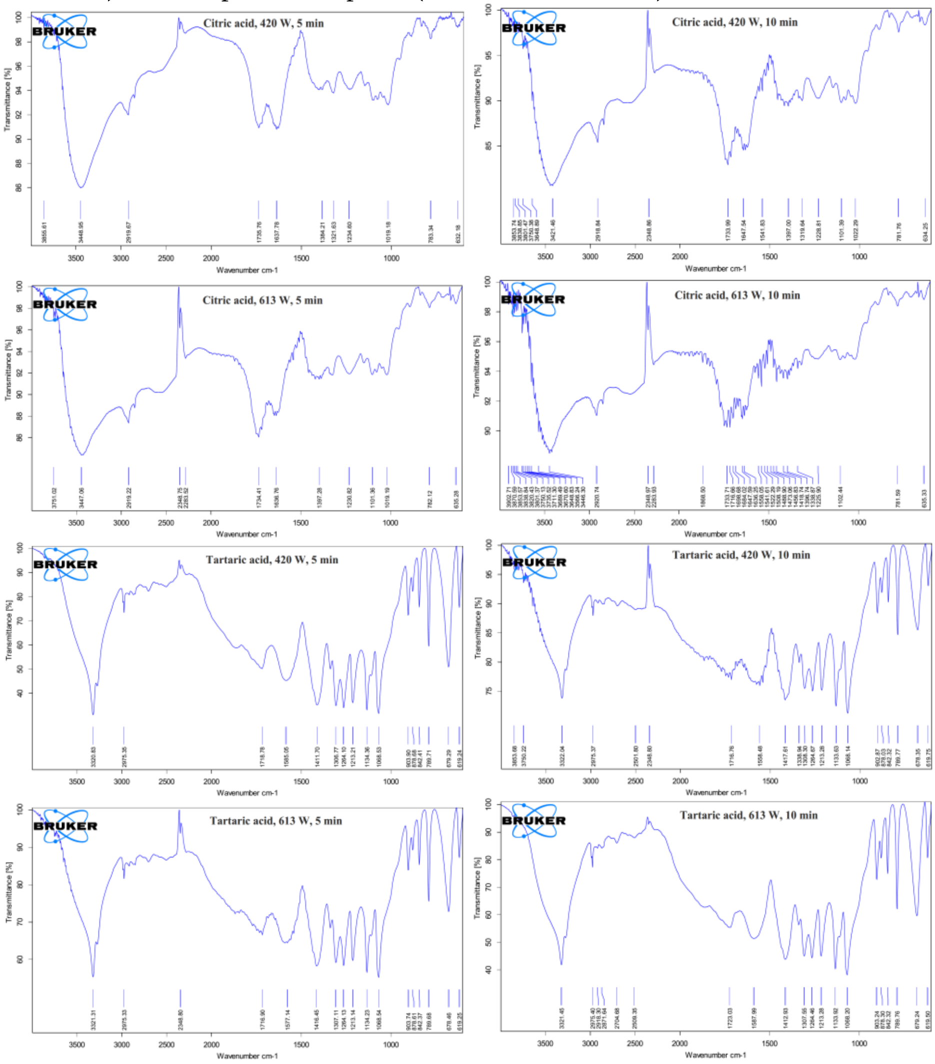

Figure 1. The FT-IR spectra of BPP obtained using various extraction conditions

Morphology of the pectins

At a low magnification, the shape of BPP particles was also similar to pectin from other materials (data not shown), such as jackfruit waste (Begum *et al.*, 2017) and *Akebia trifoliata* var. *australis* peel (Jiang *et al.*, 2012). The pectin particles had lamellate, lumpish and irregular powder shapes and a rough surface. At a higher magnification (3 000×), Figure 2 shows the structure on the surface of BPP quite clearly. The structure of pectin extracted using various solvents was significantly different. All BPP particles extracted using citric acid were significantly ruptured and more porous on the surface, while the morphology of the pectins extracted using tartaric acid was slightly ruptured and showed a slight tendency to be wrinkled at 613 W for 10 min.

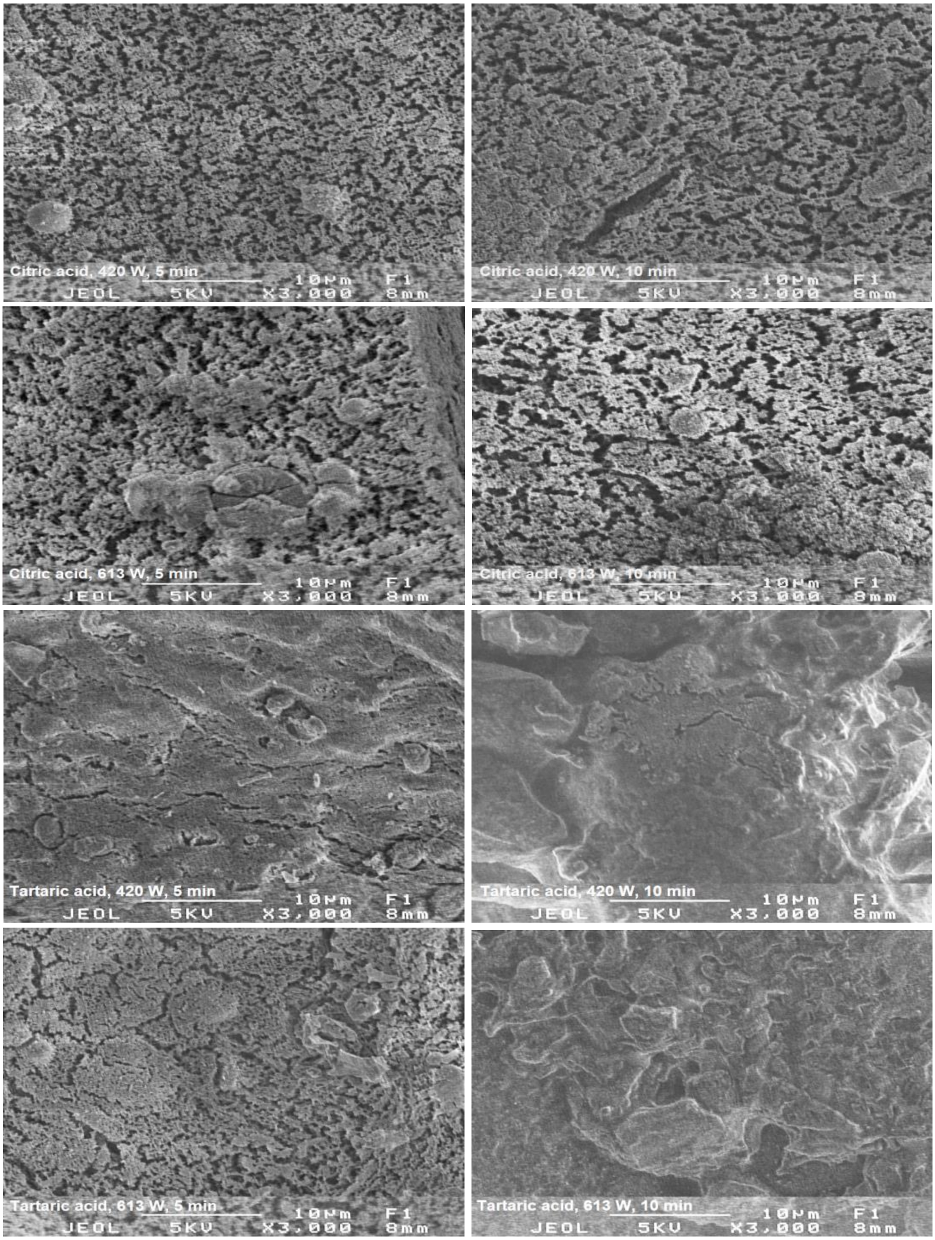

Figure 2. Scanning electron micrographs of BPP at various extraction conditions

Also, the microwave treatment caused a partially collapsed and ruptured morphology in the microstructure of the extracted pectin (Rahmati *et al.*, 2012) because of the rapid rise in temperature (Liew *et al.*, 2016) and the high microwave power can lead to loosening of the cell wall matrix (Kamal *et al.*, 2020). In addition, sources of raw materials as well as extraction conditions (pH, time, solid to solvent ratio, etc.) strongly influence the morphology of the obtained pectin.

CONCLUSIONS

In this study, pectin was successfully extracted from banana peels using MAE and citric/tartaric acid as solvents. The result revealed that the physical and chemical properties of pectin were significantly affected by the modes of extraction (solvent, microwave power and irradiation time). The results also indicated that pectin extracted from both solvents was of LMP with high purity. Besides, using different solvents make significant changes in the morphology of the pectin. The product obtained is still an ideal pectin source that can be used in the food industry in the future.

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REFERENCES

Aina, V.O., Barau, M.M., Mamman, O.A., Zakari, A., Haruna, H., Hauwa Umar, M.S., & Abba, Y.B. (2012): Extraction and characterization of pectin from peels of lemon (*Citrus limon*), grape fruit (*Citrus paradise*) and sweet orange (*Citrus sinensis*). *Br. J. Pharmacol. Toxicol.*, 3(6): 259–262.

Baississe, S., Ghannem, H., Fahloul, D., & Lekbir, A. (2010): Comparison of structure and emulsifying activity of pectin extracted from apple pomace and apricot pulp. *World J. Dairy Food Sci.*, 5(1): 79–84.

Begum, R., Yusof, Y.A., Aziz, M.G., & Uddin, M.B. (2017): Structural and functional properties of pectin extracted from jackfruit (*Artocarpus heterophyllus*) waste: Effects of drying. *Int. J. Food Prop.*, 20: 190–201.

Castillo-Israel, K.A.T., Baguio, S.F., Diasanta, M.D.B., Lizardo, R.C.M., Dizon, E.I., & Mejico, M.I.F. (2015): Extraction and characterization of pectin from Saba banana [*Musa 'saba'* (*Musa acuminata* x *Musa balbisiana*)] peel wastes: A preliminary study. *Int. Food Res. J.*, 22(1): 202–207.

Coates, J. (2000): Interpretation of infrared spectra – A practical approach. *In* R.A. Meyers, eds. *Encyclopedia of analytical chemistry*, pp. 11498–11520. Chichester, Wiley.

Emaga, T.H., Ronkart, S.N., Robert, C., Wathelet, B., & Paquot, M. (2008): Characterisation of pectins extracted from banana peels (*Musa AAA*) under different conditions using an experimental design. *Food Chem.*, 108(2): 463–471.

Food Chemical Codex (1996): *IV monographs*. Washington, National Academy Press, p. 283.

George, K.M., Ruthenburg, T.C., Smith, J., Yu, L., Zhang, Q., Anastasio, C., & Dillner, A.M. (2015): FT-IR quantification of the carbonyl functional group in aqueous-phase secondary organic aerosol from phenols. *Atmos. Environ.*, 100: 230–237.

Girma, E., & Worku, T. (2016): Extraction and characterization of pectin from selected fruit peel waste. *Int. J. Sci. Res. Publ.*, 6(2): 447–454.

Hung, P.V., Anh, M.N.T., Hoa, P.N., & Phi, N.T.L. (2020): Extraction and characterization of high methoxyl pectin from *Citrus maxima* peels using diferent organic acids. *J. Food Meas. Charact.*, 15: 1541–1546.

Jiang, Y., Du, Y., Zhu, X., Xiong, H., Woo, M.W., & Hu, J. (2012): Physicochemical and comparative properties of pectins extracted from *Akebia trifoliata* var. *australis* peel. *Carbohydr. Polym.*, 87: 1663–1669.

Kamal, Md.M., Ali, Md.R., Hossain, A., & Shishir, M.R.I. (2020): Optimization of microwave-assisted extraction of pectin from *Dillenia indica* fruit and its preliminary characterization. *J. Food Process. Preserv.*, 44(6): e14466.

Kamble, P.B., Gawande, S., & Patil, T.S. (2017): Extraction of pectin from unripe banana peel. *Int. Res. J. Eng. Technol.*, 4(7): 2259–2264.

Khamsucharit, P., Laohaphatanalert, K., Gavinlertvatana, P., Sriroth, K., & Sangseethong, K. (2018): Characterization of pectin extracted from banana peels of different varieties. *Food Sci. Biotechnol.*, 27(3): 623–629.

Koštálová, Z., Aguedo, M., & Hromádková, Z. (2016): Microwave-assisted extraction of pectin from unutilized pumpkin biomass. *Chem. Eng. Process.*, 102: 9–15.

Kratchanova, M., Pavlova, E., & Panchev, I. (2004): The effect of microwave heating of fresh orange peels on the fruit tissue and quality of extracted pectin. *Carbohydr. Polym.*, 56(2): 181–185.

Liew, S.Q., Ngoh, G.C., Yusoff, R., & Teoh, W.H. (2016): Sequential ultrasound-microwave assisted acid extraction (UMAE) of pectin from pomelo peels. *Int. J. Biol. Macromol.*, 93: 426–435.

Maran, J.P., Sivakumara, V., Thirugnanasambandhama, K., & Sridhar, R. (2013): Optimization of microwave assisted extraction of pectin from orange peel. *Carbohydr. Polym.*, 97(12): 703–709.

Mesbahi, G., Jamaliana, J., & Farahnaky, A. (2005): A comparative study on functional properties of beet and citrus pectins in food systems. *Food Hydrocolloids*, 19: 731–738.

Mohamadzadeh, J., Sadeghi-Mahoonak, A.R., Yaghbani, M., & Aalami, M. (2010): Extraction of pectin from sunflower head residues of selected iranian cultivars. *World Appl. Sci. J.*, 8(1): 21–24.

Mudgil, D. (2017): The interaction between insoluble and soluble fiber. *In* R.A. Samaan, eds. *Dietary Fiber for the Prevention of Cardiovascular Disease: Fiber's Interaction between Gut Microflora, Sugar Metabolism, Weight Control and Cardiovascular Health*, pp. 35–59. Los Angeles, Academic Press.

Nguyen, B.M.N., & Pirak, T. (2019): Physicochemical properties and antioxidant activities of white dragon fruit peel pectin extracted with conventional and ultrasound assisted extraction. *Cogent Food Agric.*, 5: 1633076.

Owens, H.S., McCready, R.M., Shepard, A.D., Schultz, T.H., Pippen, E.L., Swenson, H.A., Miers, J.C., Erlandsen, R.F., & Maclay, W.D. (1952): Methods used at Western regional research laboratory for extraction of pectic materials. *Bur. Agric. Ind. Chem.*, p. 9.

Phaiphan, A. (2019): Optimisation of pectin extraction assisted by microwave from banana (*Musa sapientum* L.) fruit peels using response surface methodology. *Carpathian J. Food Sci. Technol.*, 11(2): 127–140.

Posé, S., Kirby, A.R., Mercado, J.A., Morris, V.J., & Quesada, M.A. (2012): Structural characterization of cell wall pectin fractions in ripe strawberry fruits using AFM. *Carbohydr. Polym.*, 88: 882–890.

Quoc, L.P.T., Huyen, V.T.N., Hue, L.T.N., Hue, N.T.H., Thuan, N.H.D., Tam, N. T.T., Thuan, N.N., & Duy, T.H. (2015): Extraction of pectin from pomelo (*Citrus maxima*) peels with the assistance of microwave and tartaric acid. *Int. Food Res. J.*, 22(4): 1637–1641.

Rahmati, S., Kang, O.L., Momeni, E., & Ramli, N. (2012): Morphological studies on pectin extracted from dragon fruit peel using microwave and conventional extraction methods. *Proceedings of International Conference on Halal Gums, Penang, Malaysia*, pp. 175–180.

Ramli N., & Asmawati. (2011): Effect of ammonium oxalate and acetic acid at several extraction time and pH on some physicochemical properties of pectin from cocoa husk (*Theobroma cacao*). *Afr. J. Food Sci.*, 5(15): 790–798.

Rivadeneira, J.P., Wu, T., Ybanez, Q., Dorado, A.A., Migo, V.P., Nayve Jr, F.R.P., & Castillo-Israel, K.A.T. (2020): Microwave-assisted extraction of pectin from "Saba" banana peel waste: Optimization, characterization, and rheology study. *Int. J. Food Sci.*, ID 8879425.

Rolin, C. (1993): Pectins. *In* R.L. Whistler & J.N. BeMiller, eds. *Industrial gums: Polysaccharides and their derivatives*, pp. 257–293. San Diego, Academic Press.

Sarah, M., Hanum, F., Rizkyand, M., & Hisham, M.F. (2018): Microwave-assisted extraction of pectin from cocoa peel. *IOP Conf. Ser.: Earth Environ. Sci.*, 122: ID 012079.

Serrano-Cruz, M.R., Villanueva-Carvajal, A., Rosales, E.J.M., Dávila, J.F.R., & Dominguez-Lopez, A. (2013): Controlled release and antioxidant activity of Roselle (*Hibiscus sabdariffa* L.) extract encapsulated in mixtures of carboxymethyl cellulose, whey protein, and pectin. *LWT - Food Sci. Technol.*, 50: 554–561.

Venzon, S.S., Canteri, M.H.G., Granato, D., Junior, B.D., Maciel, G.M., Stafussa, A.P., & Haminiuk, C.E.I. (2015): Physicochemical properties of modified citrus pectins extracted from orange pomace. *J. Food Sci. Technol.*, 52(7): 4102–4112.

Wongkaew, M., Sommano, S.R., Tangpao, T., Rachtanapun, P., & Jantanasakulwong, K. (2020): Mango peel pectin by microwave-assisted extraction and its use as fat replacement in dried chinese sausage. *Foods*, 9: ID 450.

Koraqi, H., Lluga-Rizani, K. (2022): Effect of extraction solvent on bioactive compounds and antioxidant activity of Cichorium intybus L. grown in Kosovo. Agriculture and Forestry, 68 (1): 159-171. doi:10.17707/AgricultForest.68.1.09

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EFFECT OF EXTRACTION SOLVENT ON BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *Cichorium intybus* L. GROWN IN KOSOVO

SUMMARY

The aim of this study was the investigation of the total phenolic content, flavonoid, and the assessment of the antioxidant potential of *Cichorium intybus* L. flowers grown in the Kosovo region. The flowers of *Cichorium intybus* L. were collected, dried, and extracted with solvents with different polarities (water, EtOH, MeOH, EtOAc, and acetone), using the extraction method. The total phenolic and flavonoid contents were analyzed by using Folin–Ciocalteu's and AlCl3 reagents, respectively. The antioxidant activity was assessed by DPPH in vitro assay methods. The obtained results revealed variation in the content of phenolic compounds (5.2 mg GAE/gDW in water extract, 6.8 mg GAE/gDW in ethanolic extract,72.1 0.3 mg GAE/gDW in methanolic extract, 45.3 0.1 mg GAE/g DW in ethyl acetate extract,and 31.3 0.1 mg GAE/gDW in acetone extract), flavonoid (2.9 mg CE/gDW in water extract, 19.0 mg CE/gDW in ethanolic extract,30.50 mg CE/gDW in methanolic extract, 27.1 mg CE/gDW in ethyl acetate extract, and 25.4 mg CE/gDW in acetone extract), and antioxidant activity (23.0 μMolTE/gDW in water extract, 124.7 μMolTE/gDW in ethanolic extract, 152.4 μMolTE/gDW in methanolic extract, 92.7 μMolTE/gDW in ethyl acetate extract,and 107.2 μMolTE/gDW in acetone extract). *Cichorium intybus* L. flowers from the Kosovo region are also a rich source of flavonoids. Further studies are recommended to quantify and isolate the pure phytoconstituents from *Cichorium intybus* L. flowers grown in the Kosovo region which might serve as natural antioxidants application in the food and drug industry.

Keywords: *Cichorium intybus* L., total phenolic content, flavonoid, antioxidant activity DPPH

INTRODUCTION

Medicinal plants have been the source of therapeutic agents since ancient times. These plants have played an important role in the discovery of medicines

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and still, most people in developing countries rely on herbal medicines for their primary health care (Kandil *et al*., 2019).

Cichorium intybus L., is a plant of the family *Asteraceae*, commonly known as *chicory*, with its natural distribution in many countries of Europe. The flower, roots, and leaves of *Cichorium intybus* L. have been used traditionally for human consumption. The gastronomic uses and medical properties of *Cichorium intybus* L. have been well known for a long time (Jancic *et al*., 2017).

Food vegetables are a significant part of the human diet worldwide since ancient times. In this context, the Mediterranean diet is recognized as a valuable source of health benefit constituents and represents one of the most peculiar examples of traditional cuisine with many dishes rich in healthy vegetables. The traditional recommendation for a healthy diet with salad vegetables including *Cichorium intybus* L. is relevant as a dietary source of natural antioxidants, which have been associated with a lower risk of cardiovascular diseases and cancer (Tardugno *et al*., 2018, Sinkovic *et al*., 2014).

Cichorium intybus L. has gained attention for its content of phytochemicals with nutraceutical potential. Phytochemicals, the plant-derived non-nutritive compounds, are one of the different types of dietary factors which play an important role in various functions of the human body (Lee *et al*., 2017). All parts of this plant possess great importance due to the presence of compounds with health benefits, such as phenolic acids, flavonoids, coumarin, cinnamic acid derivatives, and anthocyanins, alkaloids, inulin, sesquiterpene lactones, vitamins, chlorophyll pigments, unsaturated sterols, saponins, and tannins (Sahan *et al*., 2017, Abbas *et al*., 2015).

Chicory is widely used in herbal preparations which display a beneficial influence on bile excretion, diuretic action, gastric juice excretion, as well as stimulation of digestion and metabolism of food ingredients (Milala *et al*., 2009).In the last decades, an increasing interest has been observed in the health-promoting properties of particular food constituents, including dietary fiber-polyphenolic complex (Juskiewicz *et al*., 2011). Other health benefits of *Chicorim intybus* L. as anti-microbial, anti-inflammatory, anti-mutagenic, anticarcinogenic, anti-toxic, anti-hyperglycemic, anti-ulcerogenic activities, easing digestive problems and heartburn, reducing arthritis complaints and reducing the risk of liver, as well as supporting the immune system (Sahan *et al*., 2017).

An important class of natural bioactive compounds is represented by polyphenols which are considered secondary metabolites synthesized by plants, vegetables, and medical plants in their normal development and also as a response to stress factors (Cisneros-Zhevallos *et al*., 2020). As polyphenol compounds, flavonoids, and phenolic acids are natural antioxidants that have important roles in protecting biological systems against the harmful consequences of oxidative stress. A valuable source of polyphenolic compounds is the plant species from the Asteraceae family including *chicory* (Epure *et al*., 2021). They are characterized by aromatic rings and hydroxyl groups, and they can be divided into several classes, such as phenolic acids, stilbenes, flavonoids, and lignans (Sinkovic *et al*., 2015). The great importance of phenolic compounds in the various physiological and morphological features, such as defense mechanisms, cell wall structure, interaction with phytohormones, proteins, and enzymes, with a high potential to neutralize free radicals by donating the electrons and signaling for gene expression (Petropoulos *et al*., 2017, Zhao *et al*., 2014).

Flavonoids, a subfamily of polyphenols compounds, exhibit several interesting biological, exert a wide range of biochemical and pharmacological effects that add to their well-known antioxidant capacity such: antibacterial, hepatoprotective, anti-inflammatory, anti-allergic, anti-cancer, and antiviral (Becker *et al*., 2016).Many studies revealed that antioxidant capacity is attributed to a combination of polyphenols and flavonoids (Balea *et al*., 2018), as was considered in our study because the total polyphenolic content and flavonoids content of the *Cichorium intybus* L. extracts was correlated with the antioxidant effects.

The chemical structure of the phenolic compounds and their concentration is related to the antioxidant capacity of the extracts. The mixture of phenolic compounds present in extracts can determine different supplementary effects such as synergistically, additively, or antagonistically actions, that influence the total capacity of the extract to neutralize free radicals. Both phenolic acids and flavonoids are known as antioxidant compounds, but their ability depends not only on the quantities but on the chemical structure also (Jacobo-Velázquez *et al*., 2009).

Antioxidant properties of *Cichorium intybus* L. have previously been reported in methanolic, water/acetone, and aqueous extracts, as also within the hydroalcoholic extract (Tusevski *et al*., 2013, Eray *et al*., 2020, Denev *et al*., 2014). Extraction of bioactive compounds from plant materials gains more and more interest within the food and drug industry. Extraction yield and antioxidant activity depend on the extraction method also the solvent used for the extraction process (Do *et al*., 2014). The presence of various antioxidant compounds with different chemical characteristics and polarities may or might not be soluble in a particular solvent. Polar solvents are frequently used for recovering polyphenols from plant matrices. The foremost suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Sultana *et al*.,2009). Ethanol has been referred to as a suitable solvent for polyphenol extraction and is safe for human consumption. Methanol has been generally found to be more efficient in the extraction of lower relative molecular mass polyphenols, whereas aqueous acetone is suited for the extraction of high molecular mass flavanols (Do *et al*., 2014).

Ethnobotanical studies show that the medical plants still play a crucial role in the sphere of human health in Kosovo, especially in isolated rural areas (Mustafa and Hajdari, 2014).

Cichorium intybus L. preparation as infusion and decoction form, still consumed for the prevent hart disorders, atherosclerosis, bronchitis, urinary system infections, anti-haemorrhoid, and hepatic disorders (Mustafa and Hajdari, 2014).

According an inventory of MAP (Medicinal and Aromatic Plants) and WB (Wild Berries) growing spontaneously in the territory of Kosovo,568.600 kg *Cichorium Intybus* L. grows wild in Kosovo (Millaku F., 2010).

Over the last few years, several studies have explain chemical composition and antioxidant activity of *Cichorium intybus* L. grown in Balkan Peninsula such: Denev *et al*., 2014; Dzharov *et al*., 2016, Bulgaria; Petropoulos *et al*., 2017, Greece; Epure *et al*., 2021, Romania; Jancic *et al*., 2017, Montenegro; Sinkovic *et al*., 2015, Slovenia; Tusevski *et al*.,2013, North Macedonia. According with our knowledge no any study in Kosovo about chemical composition and antioxidant activity of *Cichorium intybus* L.grown in Kosovo. Only previously studies are Faiku *et al*., 2016 about antibacterial activity of different solvent extract of *Cichorium intybus* L. grown in Kosovo.

This is the first research of this type in Kosovo and it should give us a novel result of the *Cichorium intybus* L. as a richest and cheapest source of antioxidants.

The aim of this study was the investigation of the effect of extraction solvent on major antioxidant phenolic compounds such: phenolic compounds, flavonoids, and antioxidant activity by DPPH in the extract of the *Cichorium intybus* L. grown in the Kosovo region.

MATERIAL AND METHODS

Reagents

Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteu, $AlCl_3$, $NaNO_2$, ethanol, methanol, ethyl acetate-EtOAc, acetone) and standard compounds, Gallic acid, Catechin, Trolox were purchased from Sigma-Aldrich Chemie GmbH, Germany. All reagents were of analytical grade.

Preparation of plant extracts

The *Cichorium intybus L*. flowers of about 250 g were collected from growing wild in Kosovo region within the month of July 2019. Locality was a location on central part of Kosovo (Lipjan 42° 31' 28.79" N, 21° 08' 11.40" E, elevation 559m). Voucher specimens (EE/2019/003) were deposited in the herbarium of the Laboratory of Botany in UBT-Higher Education Institution.

The flowers of *Cichorium intybus L*. after washing under running water were dried at room temperature. The flowers of the plant (1g) were ground and soaked in 30 mL of boiling water, and 30 mL organic solvents (EtOH, MeOH, EtOAc, Acetone). Then 20 mL water and 20 mL organic solvents were added sequentially during a bottom flask and soaked on an ultrasonic bath for 10 minutes.

Figure 1. *Cichorium intybus* L. growing wild in the Kosovo region

Table 1. Location information of the regions that samples were collected

No.	Locality	Latitude	Longitude	Elevation (m)	Voucher specimens no.
1	Lipjan	42° 31' 28.79" N	21° 08' 11.40" E	559 m	EE/2019/003

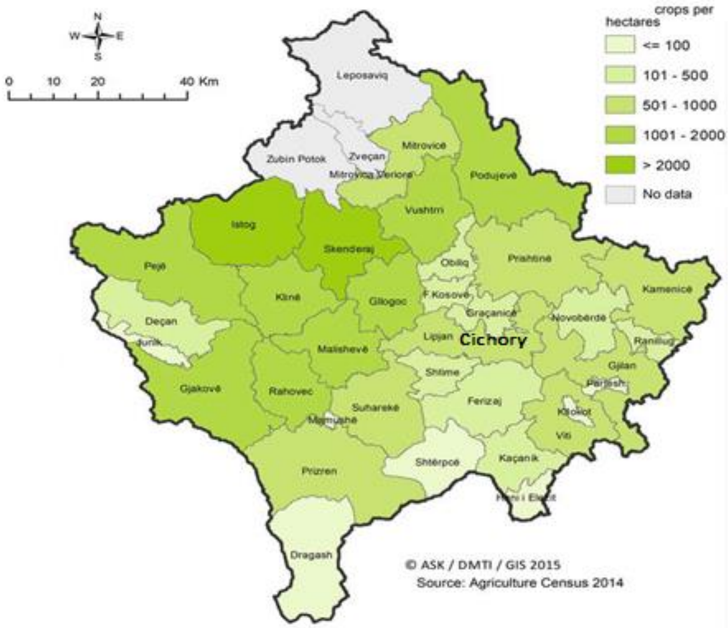

Figure 2. Map of the area where sample collection.

Determination of total phenolic content

The total phenolic content of the different extracts (water,EtOH, MeOH, EtOAc, Acetone) was estimated according to the previously reported method Singleton *et al*., (1999) and slight modifications, with the help of the Folin-Ciocalteu reagent. 200 µL of extract or standard gallic acid (5–100 mg L^{-1}) was added to 3 mL distilled water and then 250 µL Folin-Ciocalteu reagent/2 min was added. Then 750 µL sodium carbonate 20% was added and the mixture was made up to 5 mL with distilled water. The mixture was incubated for 2 h and the absorbance was measured at 765 nm in a GENESYS™ 10S spectrophotometer. Total phenolic content was expressed as gallic equivalent (mg GAE/g DW). Standard Gallic acid (GA) in a concentration of 5-100 µg/mL was used to construct the external calibration curve.

Determination of total flavonoid content

The total flavonoid content of the different extracts (water, EtOH, MeOH, EtOAc, Acetone) was estimated by colorimetric assay with the $AlCl_3$ method by Montefusco *et al*., (2015) with some modifications. On 1 mL aliquots of the samples added 2 mL distilled water and 0.3 mL of 2% $NaNO_2$. After 5min, 0.5 mL of 1% $AlCl_3$ was added, followed, after a further 6 min, by 2 mL of 1M NaOH and 2 mL of distilled water. The absorbance was read at 510 nm in a GENESYS™ 10S spectrophotometer. Catechin was used as a standard and the flavonoid content was expressed as g catechin equivalent g^{-1} dry weight of plant material (mg CE/g DW).

Antioxidant activity

Antioxidant activity of the different extracts (water, EtOH, MeOH, EtOAc, Acetone) was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazil radical) assay Brand-Williams *et al*., (1995) and Petropoulos *et al*., (2017) with some modifications. A brief description of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was dissolved in methanol to prepare a 100 µM working standard solution. To 1 mL of various concentrations of the different extract (25, 50, 100, and 200 µg/mL) 2 mL of DPPH solution was added and the resulting mixture was mixed well. After incubation in dark at room temperature for 45 min, the absorbance of these solutions was measured at 515nm in a GENESYS™ 10S spectrophotometer. $A_{control}$ was prepared in a similar manner by replacing the amount of sample with methanol.

% Inhibition of DPPH radical was calculated using the following formula:

$$\% \text{ inhibition of DPPH} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where; $A_{control}$ and A_{sample} are absorbance of control and sample, respectively. All determinations were performed in triplicates.

Statistical Analysis

For all the analyses, three samples were analyzed for each treatment and all of the assays were carried out in triplicate. The results were expressed as mean values and standard deviations (Mean ± SD). Statistical analysis of data was applied using SPSS v. 22.0 program through a one-way analysis of variance (ANOVA) while, for means where a statistical difference was detected, means comparisons were carried out using Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Total phenolic content

The total phenolic content of both extract-water and organics extract (EtOH, MeOH, EtOAc, Acetone) from flower of the *Cichorium intybus* L. was analyzed using the Folin–Ciocalteu method are shown in table 2,and figure 3a and 4 which ranged from 5.2± 0.2 mg GAE/g in water extract, 6.8± 0.2 mg GAE/g in ethanolic extract, 72.1± 0.3 mg GAE/g in methanolic extract, 45.1± 0.3 mg GAE/g in ethyl acetate extract, and 31.3± 0.2 mg GAE/g in acetone extract. From our results, we showed the methanolic extract gave higher total phenolic content compared to those water, and other organics solvent extract ($p > 0.05$). The lowest amount of the total phenolic contents was determined in the water extracts (5.2± 0.2 mg GAE/g). The total phenolic content of the water extract is significantly less than that of other organics solvents ($p < 0.05$). The total phenolic contents in water and organic solvents are ranged: Water<EtOH<Acetone<EtOAc<MeOH. Our results correspond with the results of the work Morales *et al*.,(2014), which reported that the total phenolic content of flower extract of *Cichorium intybus L*. in methanolic extract in the range from 51.1± 0.8 mg GAE/g. Tusevski *et al*.,(2013) reported that the total phenolic content of *Cichorium intybus L*. in methanolic extract range from 33.36± 0.14 mg GAE/g. Our results were the higher of these studies. Piluzza *et al*.,(2014) reported a higher amount of total phenolic compounds in the acetone/water (7:3) extract of *Cichorium intybus* L. range about 33.76 to 43.89 g GAE/Kg.Our results were the lowest of these studies. Eray *et al*.,(2020) reported total phenolic content in herbal parts of *Cichorium intybus* L.water extract range about 138.8±0.87 µg GAE/mg,and methanolic extract range about 186.3±3.281 µg GAE/mg. Also, our results were the lowest of these studies.The other hand Denev *et al*.,(2014) reported that the total phenolic content of *Cichorium intybus* L. in water extract range from about 3.7± 0.4 mg GAE/g to 6.7± 0.9 mg GAE/g, and in ethanolic extract range about 4.3± 0.5 mg GAE/g. Our results were the higher of these studies. In EtOAc extract no have any previously publication results (EtOAc=No).

Total Flavonoids content

The total flavonoids content of both extract-water and organics extract (EtOH, MeOH, EtOAc, Acetone) from flower of the *Cichorium intybus* L. analyzed using the $AlCl_3$ method by spectrophotometry are shown in table 2,and figure 3b and 4 which ranged from 2.9± 0.2 mg CE/g in water extract, 19.0± 0.3 mg CE/g in ethanolic extract, 30.5± 0.2 mg CE/g in methanolic extract, 27.1± 0.3 mg CE/g in ethyl acetate extract, and 25.4± 0.2 mg CE/g in acetone extract. From our results, we showed the methanolic extract gave higher total flavonoid content compared to those water, and other organics solvent extracts ($p > 0.05$). The lowest amount of the total flavonoid contents was determined in the water extracts (2.9± 0.2 mg CE/g). The total flavonoid content of the water extract is significantly less than that of other organics solvents ($p<0.05$). The total flavonoid contents in water and organic solvents are ranged: Water<EtOH<Acetone<EtOAc<MeOH. Our results correspond with the results of the work of Denev *et al.*,(2014), which reported that the total flavonoid content of flower extract of *Cichorium intybus* L. in water extract in the range from 2.7± 0.2 mg CE/g to 2.8± 0.2 mg CE/g. Tusevski *et al.*,(2013) reported that the total flavonoid content of *Cichorium intybus* L. in methanolic extract range from 8.06± 0.24 mg CE/g. Our results were the higher of these studies.The lowest values of total flavonoids content were obtained by Malik *et al.*,(2017) on methanolic extract (13.5± 0.70 mg RE/g) and ethanolic extracts (8.49± 0.08 mg RE/g DW.) from chicory leaves. Piluzza *et al.*,(2014) reported a higher amount of total flavonoid compounds in the acetone/water (7:3) extract of *Cichorium intybus* L. range about 27.13 to 36.94 g CE/Kg.Our results were the lowest of these studies. Eray *et al.*,(2020) reported total flavonoid content in herbal parts of *Cichorium intybus* L.water extract range about 388.3±5.77 µg CE/mg, and methanolic extract range about 550±14.53 µg CE/mg. Also, our results were the lowest of these studies.On the other hand, Kandil *et al.*,(2019) reported that the total flavonoid content of *Cichorium intybus L.* in methanolic extract range about 167.47 ± 5.83 µg QE/g,whiles in ethanolic extract range about 36.56 ± 5.95 µg QE/g.Our results were the higher of these studies.Also,the assessment flavonoid in EtOAc extracts does not have any previously publication results (EtOAc=No).

Antioxidant activity by DPPH

DPPH free radical scavenging method is a widely used and reliable method to evaluate the *in vitro* antioxidant activity of natural products and plant extracts. The natural or synthetic antioxidants such as ascorbic acid, tocopherol, cysteine, glutathione, gallic acid, etc., have the ability to reduce the DPPH radial (purple color) to a yellow-colored compound. The extent of color change depends on the hydrogen donating ability of the antioxidants.

Therefore, in the current study, we have used the DPPH method to evaluate the antioxidant activity of the *Cichorium intybus* L. flowers extracts. All extracts of the *Cichorium intybus* L. flowers exhibited higher antioxidant activity. In this study, the extracts of the undertaken of the *Cichorium intybus L.* flowers extract were assessed for antioxidant potential by utilizing the above principle of the DPPH radical scavenging method. Table 2 and figure 3c and 4 represents the DPPH radical scavenging abilities of the *Cichorium intybus L.* flowers extract used in this study. The antioxidant activity of the extract (water and organics solvent extract) from the flower of the *Cichorium intybus L.* was analyzed using the DPPH method, and are shown in table 1, and figure 3 which ranged from 23.0± 0.4 µMolTE/g in water extract, 124.7± 0.2 µMolTE/g in ethanolic extract, 152.4± 0.3 µMolTE/g in methanolic extract, 92.7± 0.3 µMolTE/g in ethyl acetate extract, and 107.2± 0.4 µMol TE/g in acetone extract. From our results, we showed the methanolic extract (152.4±0.3µMolTE/g) gave higher antioxidant activity content compared to other extracts. A good correlation was observed between total phenolic content and antioxidant activity. It is observed from the results of this study that the highest phenolic and flavonoid content of chicory extracts exhibited high antioxidant activities (Kandil *et al*.,2019). The higher DPPH antioxidant activity in methanolic extract of *Cichorium intybus* L. is significantly higher than that of other solvents ($p > 0.05$). The DPPH antioxidant activity of the water extract is significantly less than that of other solvents ($p < 0.05$).

Previous publications found that the methanolic extract of *Cichorium intybus L*. flowers possessed the highest content of phytochemicals and antioxidant activity. Antioxidant activity obtained by organic solvents are ranged:water<EtOAc<Acetone<EtOH<MeOH. Our results of the antioxidant activity content as compared to those reported by Tusevski *et al*., (2013), reported that the total antioxidant activity of *Cichorium intybus* L.flower in methanolic extract range about 154.40± 0.2 µM TE/g. Similar results also obtained Epure *et al*.,(2021), they reported the DPPH radical scavenging activity of the methanolic extracts 164.98± 5.93 µg/mL and 336.35± 11.77 µg/mL for the ethanolic extract.Denev *et al*.,(2014), reported that the DPPH antioxidant activity of *Cichorium intybus* L. flower in water extracts range about 16.1± 2.8 mM TE and 29.1± 0.8 mM TE. The DPPH antioxidant activity of *Cichorium intybus* L. flower in ethanolic extracts range about 29.2± 0 mM TE and 31.3± 0.1 mM TE. Our results were the lowest of these studies. On the other hand, Piluzza *et al*.,(2014) reported the total antioxidant activity by DPPH in the acetone/water extract of *Cichorium intybus* L. flower range about 15.63 to 23.86 mMol/g.Our results also were the lowest of these studies.The results of the study of the total antioxidant activity by DPPH in the water extract,and methanolic extract of *Cichorium intybus* L. flower presented Eray *et al*.,(2020),they reported the total antioxidant activity by DPPH calculation as IC_{50} in the water extract range about 7.5± 0.1, whiles in the methanolic extract range about 3.59± 0.18. Our results were the highest of these studies.Not found any previous publication about total antioxidant activity by DPPH in EtOAc.

Until now, no any detailed information was available for antioxidant activity of *Cichorium intybus* L. flowers grown on the Kosovo region. This is the first research about total phenolic content, total flavonoid content, and antioxidant activity of *Cichorium intybus* L. flowers grown on the Kosovo region. In other view point the best solvent for extraction biological active compounds such: phenolic content, flavonoid content, and antioxidant activity is methanol. From results obtained in this study, different extracts of *Cichorium intybus* L. flowers can be considered a potential source of antioxidants for food and drug industry application.

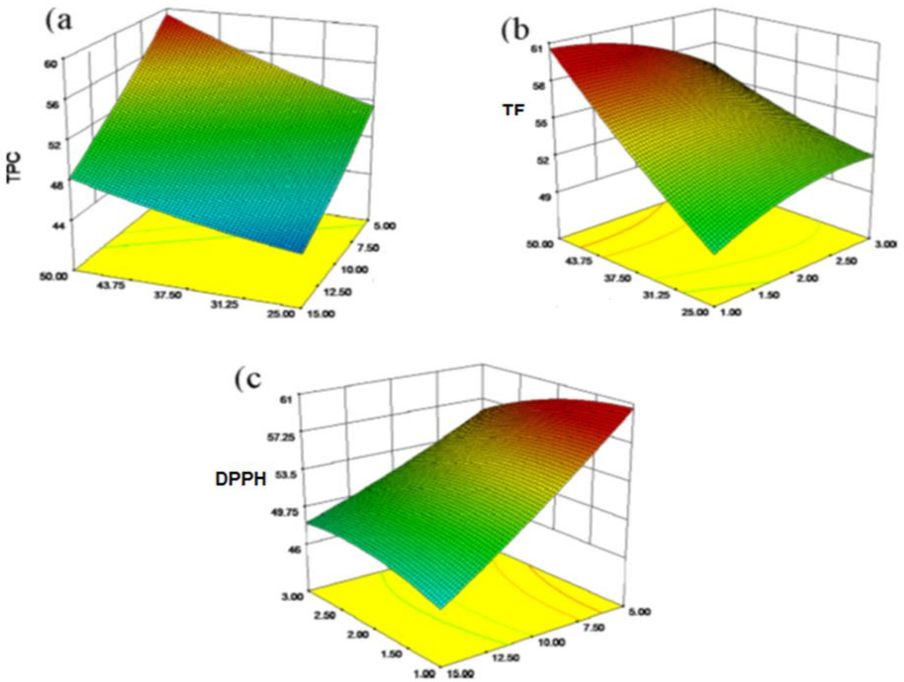

Figure 3. 3D visualization of the a) total phenolic contents b) total flavonoids contents and c) Antioxidant activity by DPPH method of *Cichorium intybus* L. extracts on different solvents

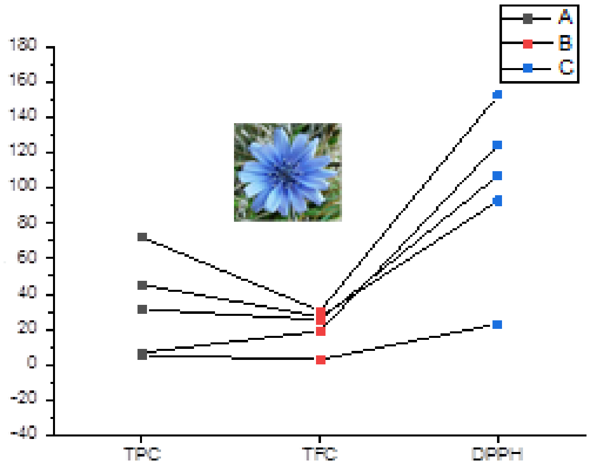

Figure 4. Correlation between total phenolic contents, total flavonoids contents, and Antioxidant Activity of *Cichorium intybus* L. extract on different solvents

Table 2. The total phenolic contents, total flavonoid content, and Antioxidant activity DPPH of *Cichorium intybus* L. extracts

Extracts	Total phenolic content (mg of GAE/gDW)	Total flavonoids content (mg of CE/gDW)	Antioxidant activity DPPH (µMolTE/gDW)
Water	5.2±0.2	2.9±0.2	23.0±0.4
EtOH	6.8±0.2	19.0±0.3	124.7±0.2
MeOH	72.1±0.3	30.5±0.2	152.4±0.3
EtOAc	45.1±0.3	27.1±0.3	92.7±0.3
Acetone	31.3±0.2	25.4±0.2	107.2±0.4

[1] Data are presented as average value ± standard deviation of three replicates

CONCLUSIONS

This study is currently the first comprehensive report that presented detailed information for phenolic content, flavonoids content, and antioxidant activity of *Cichorium intybus* L. flowers extract grown in the Kosovo region that has been examined. Very limited information is available on compositional and health-enhancing properties of *Cichorium intybus* L. grown in the Kosovo region. The result showed that *Cichorium intybus* L. flowers extract had a higher content of total phenolic contents and higher content of total flavonoids, also antioxidant activity. The highest total phenolic and flavonoid contents were determined in the flower methanolic extract. The results of our study indicate the presence of major classes of phytochemicals in *Cichorium intybus* L. flowers extract and a direct relationship between antioxidant capacities and total flavonoid content in *Cichorium intybus* L. flowers extract. Further studies are recommended to quantify and isolate the pure phytoconstituents from *Cichorium intybus* L. flowers extract which might serve as the cheapest source of natural antioxidants application in the food and drug industry. In conclusion, chicory, due to its phenolic and antioxidant contents as well as the bio accessibility of these compounds, provides important health benefits for consumers, while remaining an inexpensive vegetable. However, before this product is incorporated into a dietary supplement or as a natural food antioxidant, it is important to further study toxicity and in vivo activity.

REFERENCES

Abbas, Z.K., Saggu, S., Sakeran, M.I., Zidan, N., Rehman, H.& Ansari, A.A. (2015): Phytochemical, antioxidant and mineral composition of hydroalcoholic extract of chicory (*Cichorium intybus* L.) leaves. Saudi J. Biol. Sci. 22, 322–326.

Balea¸ S.S., Pârvu, A.E., Pop, N., Marín, F.Z.& Pârvu, M. (2018): Polyphenolic Compounds, Antioxidant, and Cardioprotective Effects of Pomace Extracts from Feteasca Neagra Cultivar. Hindawi Oxidative Medicine and Cellular Longevity. Volume 2018.

Becker, L., Zaitera, A., Petit, J., Zimmer, D., Karam, M.C., Baudelairec, E., Scherb, J.& Dicko, A. (2016): Improvement of antioxidant activity and polyphenol content of Hypericum perforatum and Achillea millefolium powders usingsuccessive grinding and sieving, Industrial Crops and Products 87; 116–123.

Brand-Williams, W., Cuvelier, M.E.& Berset, C. (1995): Use of a free radi¬cal method to evaluate antioxidant activity. Lebensm. Wiss. Tech¬nol., 28, 25–30.

Cisneros-Zevallos, L., Jacobo-Velázquez, D.A. (2020): Controlled Abiotic Stresses Revisited: From Homeostasis through Hormesis to Extreme Stresses and the Impact on Nutraceuticals and Quality during Pre- and Postharvest Applications in Horticultural Crop. J. Agric. Food Chem., 68, 11877–11879.

Denev, P., Petkova, N., Ivanov, I., Sirakov, B., Vrancheva, R. & Pavlov, A. (2014): Determination of biologically active substances in taproot of common chicory (*Cichorium intybus* L.). Scientific Bulletin. Series F. Biotechnologies. XVIII, 2285-1364.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, L.P., Lien Huong Huynh, L.H., Soetaredjo, F.E., Ismadji, S.&Yi-Hsu, J. (2014): Effect of extraction solvent on total phenol content,total flavonoid content, and antioxidant activity of *Limnophila aromatica*, Journal of food and drug analysis 22; 296 -302.

Dzharova V.V., Mishrab A.P., Shariatic M.A., Atanassovad M.S., &. Plygunc S. (2016): PHYTOCHEMICAL CONTENTS IN SOLID–LIQUID EXTRACTION OF AQUEOUS ALCOHOLIC EXTRACT OF CHICORY (CICHORIUM INTYBUS L.) LEAVES. Foods and Raw Materials 4;2

Epure, A., Pârvu, E.A., Vlase, L., Benedec, D., Hanganu, D., Gheldiu, M.A.,Toma, A.V.& Oniga I.(2021): Phytochemical Profile, Antioxidant, Cardioprotective and Nephroprotective Activity of Romanian Chicory Extract. Plants, 10, 64.

Eray, N., Kartal, I.D.& Ḉelik I. (2020): Antioxidant Properties of Cichorium intybus L. (Chicory) Extracts and Their Cytotoxic Effects on HepG2 Cells. YYU Journal of Agricultural Science 3,444-453.

Faiku F., Haziri A., Mehmeti I., Bajrami D. & Haziri I. (2016): EVALUATION OF ANTIBACTERIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF CICHORIUM INTYBUS (L.) GROWING WILD IN EAST PART OF KOSOVO.The Journal of Animal & Plant Sciences 26(5); 1486-1491.

Jacobo-Velázquez, D.A.& Cisneros-Zevallos, L. (2009): Correlations of antioxidant activity against phenolic content revisited: A new approach in data analysis for food and medicinal plants. J. Food Sci., 74, 107–113.

Jancic, D., Todorovic, V., Sircelj, H., Dodevska, M., Beljkas, B., Znidarcic, D. & Sobajic, S,(2017): Biologically active compounds and antioxidant capacity of cichorium intybus L. leaves from Montenegro. Ital. J. Food Sci.,29, 627.

Juskiewicz, J., Zdunczyk, Z., Zary-Sikorska, E., Krol, B., Milala, J.& Jurgonski A. (2011): Effect of the dietary polyphenolic fraction of chicory root, peel, seed and leaf extracts on caecal fermentation and blood parameters in rats fed diets containing prebiotic fructans British Journal of Nutrition, 105, 710–720.

Kandil, S.A., Elella, A.F. & Shemy, E.A.H. (2019): Cytotoxic profile activities of ethanolic and methanolic extracts of chicory plant (*Cichorium intybus* L.). Journal of Radiation Research and Applied Sciences. 12(1),106-111.

Lee,T.M.,Lin, C.W.,Yu, B.& Lee, T.T (2017): Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals — A review. Asian-Australas J Anim Sci,30 (3),299-308.

Malik, B., Pirzadah, T.B., Tahir, I.& Rehman, R.U. (2017): Chemo-profiling, Antioxidant Potential and Ionomic Analysis of Cichorium intybus L. Pharmacogn. J. 9, 917–928.

Milala, J., Grzelak, K., Król, B., Juśkiewicz, J. & Zduńczyk Z. (2009): Composition and properties of chicory extracts rich in fructans and polyphenols. Pol. J. Food Nutr. Sci. 59, 35-43.

Millaku F. (2010) Inventory of medicinal and aromatic plants and wild berries in Kosovo. (Report). Availabe online at: http://www.helvetas-ks.org/HPK/documentation/technical_reports/101007

Montefusco,A., Semitaio,G., Marrese,P.P, Iurlaro, A.,De Caroli, M., Piro,G., Dalessandro,G.& Lenucci, S.M.(2015): Antioxidants in Varieties of Chicory (*Cichorium intybus* L.) and Wild Poppy (*Papaver rhoeas* L.) of Southern Italy. Journal of Chemistry, 8 pages.

Morales, P., Ferreira, C.F.R.I., Carvalho, M.A., Sanchez-Mata, C., Camara, M., Fernandez-Ruiz, V., Pardo-de-Santayana, M.& Tardio, J. (2014): Mediterranean non-cultivated vegetables as dietary sources of compounds with antioxidant and biological activity. LWT - Food Science and Technology 55,389-396.

Mustafa B., Hajdari A. (2014): Medical ethnobotanical studies in Kosovo.In: Pieroni A, Quave CL (eds) Ethnobotany and Biocultural Diversities in the Balkans. Springer Science+Business Media New York.

Petropoulos, A.S., Fernandes, A., Barros, L. & Ferreira I. (2017): A comparison of the phenolic profile and antioxidant activity of different *Cichorium spinosum* L. ecotypes. J Sci Food Agric. 98,183–189.

Piluzza, G., Sulas, L. & Bullitta S. (2014): Dry matter yield, feeding value, and antioxidant activity in Mediterranean chicory (*Cichorium intybus* L.) germplasm. Turk J Agric For 38,506-514.

Sahan, Y., Gurbuz, O., Guldas, M., Degirmencioglu, N.& Begenirbas, A. (2017). Phenolics, antioxidant capacity and bioaccessibility of chicory varieties (*Cichorium* spp.) grown in Turkey.Food Chemistry, 217, 483–489.

Singleton, V.L., Orthofer, R.&Lamuela-Raventós R.M. (1999): Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, Methods in Enzymology 299, 152-178.

Sinkovic, L., Demšar, L., Znidarcic, D., Vidrih, R., Hribar, J.& Treutter D. (2015): Phenolic profiles in leaves of chicory cultivars (*Cichorium intybus* L.) as influenced by organic and mineral fertilizers. Food Chemistry, 166,507–513.

Sinkovic, L., Hribar, J.& Vidrih, R. (2014): Influence of Cultivar and Storage of Chicory (*Cichorium intybus* L.) Plants on Polyphenol Composition and Antioxidative Potential. Czech J. Food Sci. 32, 10–15.

Sultana, B., Anwar, F. & Ashraf, M. (2009): Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts, Molecules, 14, 2167-2180.

Tardugno, R., Pozzebon, M., Beggio, M., Turco Del, P.& Pojana G. (2018): Polyphenolic profile of *Cichorium intybus* L. endemic varieties from the Veneto region of Italy. Food Chemistry 266,175-182.

Tusevski, O., Kostovska, A., Iloska, A., Trajkovska, L.& Simic, G.S. (2014): Phenolic production and antioxidant properties of some Macedonian medicinal plants. Cent. Eur. J. Biol. 9(9), 888-900.

Zhao, H., Zhang, H. & Yang, S. (2014): Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube. Food Science and Human Wellness 3,183–190.

Savić. N. (2022): Condition factor and length-weight relationship of huchen (Hucho hucho) fingerlings in cultivated conditions. Agriculture and Forestry, 68 (1): 173-181. doi:10.17707/AgricultForest.68.1.10

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CONDITION FACTOR AND LENGTH-WEIGHT RELATIONSHIP OF HUCHEN (*HUCHO HUCHO*) FINGERLINGS IN CULTIVATED CONDITIONS

SUMMARY

The experiment of determining the condition factors and length-weight relationship of cultivated huchen (*Hucho hucho*) fingerlings aged 8 (beginning) to 11 months (end), was conducted in the aquaculture laboratory of the Faculty of Agriculture, University of Banja Luka for 91 days. A total of 35 huchen fingerlings of average body weight (W) (± SD) of 6.00 ± 1.74 g, total body length (TL) of 8.95 ± 0.98 cm, fork length (FL) 8.36 ± 0.90 cm and standard length (SL) 7.74 ± 0.85 cm were housed in three flow-through aquariums (65 l / aquarium). Body lengths and weights were measured once a month. The huchen fingerlings feeding was according to appetite, using commercial trout feed. The aim of this study was to determine the condition factor and the length - weight relationship of huchen (*Hucho hucho*) fingerlings in cultivated conditions. The growth of body length and weight of fingerlings was expressed by a highly positive correlation ($r^2 = 0.979$). Negative allometric growth ($b < 3$) was determined for the total observed period, with a tendency to intensify growth in the last observed period when positive allometric growth ($b > 3$) was recorded. The length-weight relationship of huchen fingerlings was calculated as $W = 0.011995L^{2.822}$. A highly positive correlation between body length (TL, FL and SL) and body weight was found. The condition factor (CF) huchen fingerlings for the whole observed period averaged 0.82.

Keywords: Condition, growth, cultivated huchen fingerlings

INTRODUCTION

Huchen (*Hucho hucho*) is a very important salmonid fish species for sport and recreational fishing. It is known as one of the largest salmonid fish species in the world (Holčik *et al*., 1988, Mikavica & Savić, 1999) and is one of the most endangered fish species inhabiting the Danube basin of Central Europe, and many populations are supported by artificial reproduction and restocking programs. Ihut et al., 2014). Huchen (*Hucho hucho*) is endemic to the Danube river basin district, where it inhabits streams and rivers with fast water flow, water temperature

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usually up to 15°C and sufficient amount of dissolved oxygen (8 – 9 mg L−1), although it can withstand adverse conditions (eg water temperature up to 22°C and up to 5 mg L−1 dissolved oxygen) (Simonović *et al.*, 2011). During the warmest months of the year (July-August), the average water temperature of rivers in Austria inhabited by huchen ranges from 12 to 18°C (Ratschan, 2014). In huchen habitats, water temperatures range from 6 to 18°C (Baensch & Riehl, 1991), and spawn at a water temperature of 6 to 10°C (Kahrimanović *et al.*, 2013). In the first year of life it reaches a total body length of about 25 cm and a body weight of about 150 g, and at the age of two it reaches a total body length of about 40 cm and a body weight of about 500 g (Pasarin, 2007).

Comparing the growth of huchen from the river and cultivated conditions, it is indicative that in the first year of life the growth is approximately the same and is classified as slow, while in the second year of life the growth of huchen in the river is faster (classified as average) compared to the cultivated conditions which is classified as slow growth (Andreji & Stráňai, 2013). This is primarily the result of differences in feed and space (Pavlík, 1998). The regression coefficient (b) provides information on fish growth (Sangun, 2007), and for most fish the expected range of the regression coefficient (b) is $2.5 < b < 3.5$ (Froese, 2006). Froese et al. (2014) state that the calculated value of a = 0.01 is a fish with a spindle-shaped elongated body. Simonović et al. (2011) state that the growth of huchen from the Drina River in the early stages of development was faster in length than the growth of the weight. After reaching the total length of 107.45 cm of the huchen, there is a faster growth of weight compared to the growth of body length, which can be seen from the b value of 2,187 in the younger stages, and in the older stages it increased to 3,910 (Simonović et al., 2011). This is confirmed by Andreji and Stráňai (2013) who state that the analysis of the growth of huchen, aged 5+ to 24+, caught from the river and from cultivated conditions shows positive allometric growth. Bajić et al. (2015) found a high positive correlation between body length and body weight ($R2 = 0.8359$ and $R2 = 0.9246$) of huchen fry from two examined groups in cultivated conditions, however, the b value is less than 3, ie negative allometric growth is present.

Condition factor (CF) is one of the ways to monitoring the impact of environmental factors on fish (Dekić et al., 2016), and within the population it also depends on various internal (genetics, developmental stages) parameters (Treer et al., 2013). When managing huchen, it is important to know that CF depends not only on environmental conditions, but also very strongly on body length. This means that low CF at certain lengths does not necessarily reflect suboptimal habitat conditions, as the growth of huchen body length is much faster than the growth of body weight during the first years of life (Treer et al., 2013). Faster growth in body length than body weight may be due to the elongated body of the huchen, compared to other salmonids (Vuković & Ivanović, 1971). Mruk and Kucheruk (2019) state that the condition factor of the huchen broodstock, aged 6+ and 7+, was the same and amounted to 0.77.

The aim of this study was to determine the condition factor and the length - weight relationship of huchen (*Hucho hucho*) fingerlings in cultivated conditions.

MATERIAL AND METHODS

The experiment of determining the factors of condition and length-weight relationship of cultivated huchen (*Hucho hucho*) fingerlings aged 8 (beginning) to 11 months (end), was conducted in the aquaculture laboratory of the Faculty of Agriculture, University of Banja Luka for 91 days. The seven months old huchen fingerlings, were obtained from the hatchery of the Sports Fishing Association Banja Luka (SRD BL). The huchen fingerlings were transported at the end of November 2020. from the SRD BL hatchery to the aquaculture laboratory of the Faculty of Agriculture, University of Banja Luka. Huchen fingerlings was housed in flowing aquariums, where it stayed for a month until the beginning of the experiment in order to adapt to the new environmental conditions. There was a total of 35 fingerlings of average weight (± SD) 6.00 ± 1.74 g, total length (TL) 8.95 ± 0.98 cm, fork length (FL) 8.36 ± 0.90 cm and standard length (SL) 7.74 ± 0.85 cm.

The huchen fingerlings are uniform in size (body weight and length) and distributed in three flowing aquariums (11, 12 and 12 individuals) with a volume of 65 l / aquarium, in which the same conditions were present. The water supply and drainage was independent for each aquarium, and there were 25 changes water / aquarium in 24 hours. The water in the aquariums is additionally aerated using an air compressor. The photoperiod was 7 and 17 hours, light (from 8 am to 3 pm) and darkness (from 3 pm to 8 am). The light sources were four 60 W bulbs, placed 70 cm above the aquarium. TL (cm), FL (cm), SL (cm) and W (g) were measured at the beginning of the experiment and once a month during the experiment. Body length (cm) was measured with an ichthyometer (accuracy 0.1 cm) and individual body weight (g) with a Kern EMB 600-2 digital scale (load capacity: 600 g; accuracy: 0.01 g). For anesthesia of individuals, just before measuring body length and weight, the anesthetic 2-phenoxyethanol was used. The water temperature during the experiment was measured daily with a digital thermometer (HANA, Miniterm HI 8751), and the pH value was periodically measured with a digital pH meter (WTW, Germany). Aquariums were cleaned once a week.

The huchen fingerlings feeding was according to appetite, using commercial trout feed. The chemical composition of the commercial feed used (feed granulation 1.9 mm) was: crude protein 50%, crude fat and oil 20%, crude fiber 0.7%, crude ash 7.5%, phosphorus 1.3% and digestible energy 19.2 MJ / kg.

Condition factor was calculated using the formula according to Ricker (1975):

$$CF = (W/L^3)*100$$

W - body weight (g), L - total length (cm).

The length-weight rrelationship of huchen fingerlings was determined on the basis of the total length (cm) and body weight (g) of individuals, according to the exponential function (Tesch, 1968):

$$W = aL^b$$

Transformed into logarithmic form:

$$\text{Log } W = \text{Log } a + b \text{ Log } L$$

W - weight of fish (g); a = regression constant; b = regression coefficient; L = total length (cm).

Statistical analysis included descriptive statistics, regression and correlation, and calculations were done through the statistical programs SPSS17 and MS Excel.

RESULTS AND DISCUSSION

Table 1 shows the average water temperatures, with minimum and maximum values, coefficient of variation and standard deviation for the three control periods. The average pH value of water during the experiment was 7.123.

Table 1. Water temperature during the experiment

Days	Water temperature ^{o}C				
	Average	SD	CV	Min	Max
0-32	12.5	0.92	7.3	10.5	14.3
33-64	12.3	1.13	9.2	9.7	13.9
65-91	12.3	0.57	4.7	11.1	13.4

Water temperatures during the experiment were similar and averaged 12.5^{o}C and 12.3^{o}C, respectively. Water temperatures were within the range of 6 - 18^{o}C reported by Baensch and Riehl (1991), but were lower than optimal (16 - 18^{o}C) in terms of growth and mortality of fry and fingerlings huchen reported by Jungwirth *et al.* (1989).

The largest variations were found in average body weight, with a trend of increasing variation from the beginning to the end of the experiment. In contrast to body weight, body length variations were significantly lower, indicating a more even increase in individual body length. The body of the huchen is very elongated, which can be seen from the research of Nikcevic *et al.* (1998) which stating that the catch of a four-month-old huchen fry, which was released into the river after swimming in a hatchery, determined an average total body length of 58.95 ± 2.33 mm and an average body weight of 2.07 ± 0.14 g.

The condition factor was the highest at the beginning, after which there was a tendency to fall slightly (Table 2). Limits of variation (minimum and maximum values, CV) of body length (cm), body weight (g) and CF of fingerlings during the experiment are shown in Table 2.

The results shown in Table 3 show that there are highly positive correlations between body lengths (TL, FL and SL) and body weight (W). The increase in body length was more intense and uniform to each other.

Table 2. Variations in body length (cm) and weight (g), and condition factor (CF) of huchen fingerlings during the experiment

Parameter	Days	0	32	64	91
	Age (months)	8	9	10	11
	Number of fish *(n)*	*(n=35)*	*(n=34)*	*(n=33)*	*(n=32)*
TL (cm)	Average	8.95	10.18	11.72	12.67
	SD	0.98	1.24	1.42	1.69
	CV	10.98	12.17	12.14	13.36
	Min	6.80	7.70	8.50	9.60
	Max	11.30	13.30	15.0	16.90
FL (cm)	Average	8.36	9.65	11.04	11.94
	SD	0.90	1.17	1.38	1.62
	CV	10.72	12.17	12.52	13.56
	Min	6.40	7.30	7.70	9.00
	Max	10.30	12.70	14.20	16.10
SL (cm)	Average	7.74	8.76	10.08	11.01
	SD	0.85	1.03	1.30	1.54
	CV	10.93	11.74	12.87	13.99
	Min	5.80	6.60	6.90	8.20
	Max	9.60	11.40	13.00	15.20
W (g)	Average	6.00	8.79	12.88	16.35
	SD	1.74	2.86	1.42	6.98
	CV	29.04	32.52	33.77	42.67
	Min	2.80	4.00	4.20	6.60
	Max	10.40	16.60	24.50	35.40
CF	Average	0.835	0.832	0.799	0.798
	SD	0.006	0.008	0.007	0.028
	CV	0.69	0.98	0.94	3.57
	Min	0.831	0.827	0.792	0.783
	Max	0.842	0.841	0.807	0.831

TL – total length (cm), FL – fork length (cm), SL – standard length (cm), W – body weight (g); CF – Condition factor.

Table 3. Correlative relationships of body length and mass (*Pearson Correlation*)

	TL (cm)	W (g)	FL (cm)	SL (cm)
TL (cm)	1	0.964**	0.998**	0.994**
W (g)		1	0.963**	0.966**
FL (cm)			1	0.993**
SL (cm)				1

TL – total length (cm); FL – fork length (cm); SL – standard length (cm); W – body weight (g).
**. Correlation is significant at the 0.01 level.

The total condition factor (CF ± SD) was quite stable and averaged 0.82 ± 0.02 (min 0.80; max 0.84), with minor variations (CV = 2.47) during the experiment. Bajić *et al.* (2015) state that by breeding huchen fry during the early juvenile phase CF was 0.688 (huchen fry of average weight 3 g) and 0.581 (huchen fry of average weight 1.1 g), and this is the result of using different feed during the initial diet of huchen (artemia salinae + factory feed for trout and gamarus sp. + fish meat). It is noticeable that in this experiment CF is higher compared to the results of Bajić *et al.* (2015), which indicates that in favorable environmental conditions, with increasing age, there is a uniform increase in the length and body weight of the huchen, as a result of which the condition factor increases. Accordingly, Treer *et al.* (2013) state that low CF of young shoots at certain body lengths does not necessarily reflect suboptimal habitat conditions, due to faster growth of fingerling body length in relation to body weight in the first years of life.

Survival of huchen fingerlings was high during the experiment (91.43%). Mortality was 8.57% (a total of 32 survived huchen and 3 died).

From the beginning to 64 days into the experiment, there was a negative allometric growth ($b < 3$) of huchen fingerlings with a positive correlation coefficient (r) of total length and body weight and coefficient of determination (r^2), according to Bajić *et al.* (2015). As the total length and body weight increased, the regression parameter b also increased (Table 4).

Table 4. Logarithmic values of total length and body weight, regression parameters, correlation and determination coefficient of huchen fingerlings by observed periods

Age (months)	Days exp.	No. fish (n)	Log TL			Log W			Regression parameter		R	r^2
			Min	Max	Average±SD	Min	Max	Average±SD	a	b		
8	0	35	0.83	1.05	0.949±0.049	0.45	1.02	0.759±0.133	-1.780	2.674	0.979	0.959
9	32	34	0.89	1.12	1.004±0.054	0.60	1.22	0.920±0.151	-1.870	2.778	0.992	0.985
10	64	33	0.93	1.18	1.065±0.054	0.62	1.39	1.083±0.162	-2.047	2.938	0.988	0.976
11	91	32	0.98	1.23	1.099±0.058	0.82	1.55	1.176±0.185	-2.200	3.072	0.967	0.935

Observed by days, the calculated regression coefficients (b) ranged within the limits stated by Froese (2006), that in most fish the expected range of the regression coefficient (b) was $2.5 < b < 3.5$. Analysis of logarithmic values of total length and body weight from 64 to 91 days revealed positive allometric growth ($b > 3$), with a correlation coefficient (r) of length and body weight of 0.967 and a coefficient of determination (r^2) of 0.935 (Table 4).

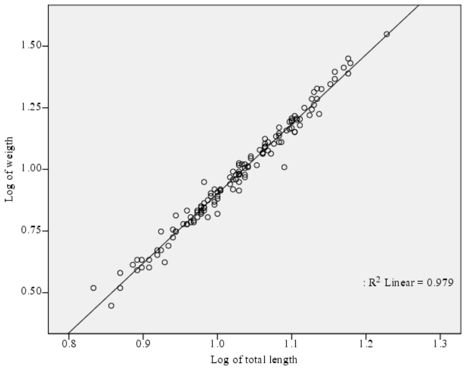
Taking into account all measured values (from the beginning to the end of the experiment), the analysis of logarithmic values of the total length and body weight of the huchen fingerlings showed a negative allometric growth ($b < 3$). The correlation coefficient (r) of total length and body weight was 0.98967, and the coefficient of determination (r^2) was 0.979 (Table 5).

Table 5. Logarithmic values of total length and body weight, regression parameters, correlation and determination coefficient of huchen fingerlings during the experiment

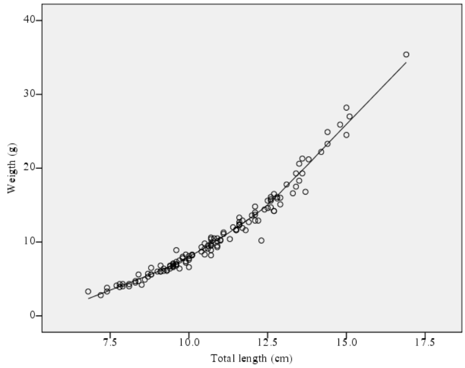
Log TL			Log W			Regression parameters		R	r^2
Min	Max	Average±SD	Min	Max	Average±SD	a	b		
0.83	1.23	1.028±0.08	0.45	1.55	0.979±0.22	-1.921	2.822	0.98967	0.979

In relation to the results of this research, Bajić *et al.* (2015) report a lower correlation of huchen fry of $R^2 = 0.9246$ and $R^2 = 0.8359$, which is related to a lower condition factor.

During the observed period, the increase in body length was more pronounced, in relation to the increase in body weight of huchen fingerlings, which was reflected in negative allometric growth ($b < 3$), according to Simonović *et al.* (2011) and Bajić *et al.* (2015). Treer *et al.* (2013) state that negative allometric growth is present in younger age categories of huchen, due to faster growth of body length in relation to body weight.



Graph 1. Logarithmic values of total length and weigth of huchen fingerlings



Graph 2. Length - weigth relationship of huchen fingerling

The equation of length-weight relationship of the analyzed huchen fingerlings is $W = 0.011995L^{2.822}$, and the logarithmic form $LogW = -1.921 + 2.822LogL$. A highly positive correlation (Graph 1) was found between the logarithmic values of the length and body weight of the huchen fingerlings in cultivated conditions ($r^2 = 0.979$).

CONCLUSIONS

The growth of body length and body weight of huchen fingerlings was expressed by a highly positive correlation ($r^2 = 0.979$). The length- weight relationship of huchen fingerlings was calculated as $W = 0.011995L^{2.822}$.

Negative allometric growth ($b < 3$) was determined for the total observed period, with a tendency to intensify growth in the last observed period when positive allometric growth ($b > 3$) was recorded.

A highly positive correlation was found between body length (TL, FL and SL) and body weight (W). The condition factor (CF) of the huchen fingerlings during the experiment averaged 0.82.

REFERENCES

Andreji, J. & Stráňai, I. (2013). Growth parameters of huchen *Hucho hucho* (L.) in the wild and under culture conditions – *Archives of Polis Fisheries* 21: pp. 179-188.

Bajić, A., Sipos, S., Pejčić, Lj., Demény, F., Sokoray-Varga, S., Müller, T. & Miljanović, B. (2015). Rearing Danube salmon, *Hucho hucho* (L. 1758), in controlled environment during early juvenile stage. *Pisces Hungarici* 9 (2015), pp. 81–88.

Baensch, H.A. & Riehl, R. (1991). Aquarien atlas. Bd. 3. Melle: Mergus, Verlag für Natur-und Heimtierkunde, Germany.

Dekić, R., Savić, N., Manojlović, M., Golub, D. & Pavličević, J. (2016). Condition factor and organosomatic indices of rainbow trout (*Oncorhynchus mykiss*, Wal.) from different broodstock. *Biotechnology in Animal Husbandry* 32 (2), Belgrade, Faculty of Agriculture, pp. 229-237, DOI:10.2298/BAH1602229D.

Froese, R. (2006): Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *Journal of Applied Ichthyology*, 22, pp. 241–253.

Froese, R., Thorson, J. & Reyes, Jr. B.R. (2014). A Bayesian approach for estimating length-weight relationships in fishes. *Journal of Applied Ichthyology* 30(1): pp. 78-85.

Holčik, J., Hensel, K., Nieslanik, J. & Skácel, S. (1988). The Eurasian Huchen, *Hucho hucho*. Largest salmon of the world – Dr. W. Junk Publishers, Dotrecht, Boston and Lancaster, p. 242.

Ihut, A., Zitek, A., Weiss, S., Ratschan, C., Holzer, G., Kaufmann, T., Cocan, D., Constantinescu, R. & Miresan, V. (2014). Danube salmon (*Hucho hucho*) in Central and South Eastern Europe: A review for the development of an international program for the rehabilitation and conservation of Danube salmon populations. Bulletin UASVM Animal Science and Biotechnologie, 71: pp. 86-101.

Jungwirth, M., Kossmann, H. & Schmutz, S. (1989). Rearing of Danube salmon *(Hucho hucho* L.) fry at different temperatures, with particular emphasis on freeze-dried zooplankton as dry feed additive. *Aquaculture*, 77: pp. 363-371.

Kahrimanović, A., Omanović, H., Halimović, S. & Kahrimanović, D. (2013). Salmonidae. Univerzitet u Sarajevu, Poljoprivredno-prehrambeni fakultet, Sarajevo.

Mikavica, D. & Savić, N. (1999). Ribe rijeke Drine [Fish of River Drina]. Univerzitet u Banjoj Luci, Poljoprivredni Fakultet, Banja Luka, p. 84.

Mruk, A. & Kucheruk, A. (2019). Fish-biological characteristics of danube salmon (*Hucho hucho* L.) breeders. International Scientific and Practical Conference; Modern technologies of propagation and restocking of native fish species, Mukachevo, Ukraine, pp. 41-42.

Nikcevic, M., Mickovic, B., Hegediš, A. & Andjus, K.R. (1998). Feeding habits of huchen *Hucho hucho* (Salmonidae) fry in the River Tresnjica, Yugoslavia, *Italian Journal of Zoology*, 65:S1, 231-233, DOI:10.1080/11250009809386821

Pavlík, L. (1998). A note to huchen stocking – *Rybarstvo*, 3: pp. 8-9.

Pasarin, B. (2007). Salmonicultură practică. Ed. Alfa pp. 69–71 Iaşi.

Ratschan, C. (2014). Aspekte zur Gefährdung und zum Schutz des Huchens in Österreich. Den 33.

Ricker, W.E. (1975). Computation and interpretation of biological statistics of fish populations. *Fisheries Research Board of Canada Bulletin* 191: pp. 1–382.

Sangun, L., Akamca, E. & Akar, M. (2007). Weight-Length Relationships for 39 Fish Species from the North-Eastern Mediterranean Coast of Turkey. *Turkish Journal of Fisheries and Aquatic Sciences* 7: pp. 37-40.

Simonović, P., Nikolić, V., Tošić, A. & Marić, S. (2011). "Length-weight relationship in adult huchen Hucho hucho (L., 1758) from Drina River, Serbia" *Biologia*, vol. 66, no. 1, 2011, pp. 156-159. https://doi.org/10.2478/s11756-010-0135-2

Tesch, F.W. (1968). Age and growth. p. 93–120. In: Ricker, W. (Ed.): *Methods for Assesment of Fish Production in Fresh Waters.* Oxford and Edinburgh.

Treer, T., Šprem, N. & Piria, M. (2013). Condition of huchen (*Hucho hucho* Linnaeus, 1758) from the Croatian-Slovenian Kupa River. Blackwell Verlag GmbH, *Journal of Applied Ichthyology*, pp. 1–3.

Vuković, T. & Ivanović, B. (1971). Slatkovodne ribe Jugoslavije/Freshwater fishes from Jugoslavia. Zemaljski muzej BiH, Sarajevo, p. 268.

Vemić, A. (2022): Occurrence and distribution of Ophiostoma novo-ulmi in "Biogradska Gora" National Park, Agriculture and Forestry, 68 (1): 183-189. doi: 10.17707/AgricultForest.68.1.11

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OCCURRENCE AND DISTRIBUTION OF *OPHIOSTOMA NOVO-ULMI* IN "BIOGRADSKA GORA" NATIONAL PARK

SUMMARY

During the monitoring of health condition in "Biogradska Gora" National Park a symptoms of Dutch elm disease were recorded. Analyses confirmed that fungus *Ophiostoma novo-ulmi* was present in symptomatic wych elm (Ulmus glabra) trees as causal agent of their wilting. This area is one of the rarest protected ecosystems of this type in the world and until now elm trees were considered to be free of Dutch elm disease. This is the first report of *Ophiostoma novo-ulmi* in "Biogradska Gora" National Park. Using genetically divergent forests as reservoirs for breathing programs against Dutch elm disease could be more difficult based on these results. Implementation of these results against Dutch elm disease on global and local scale was discussed.

Keywords: *Ulmus glabra, Ophiostoma novo-ulmi,* symptoms, Montenegro

INTRODUCTION

Forests of Biogradska Gora in Montenegro developed through natural processes, without anthropogenic disturbances and this kind of forests provide opportunity of studding close to nature management and environntal changes (Curovic *et al*., 2020; Nagel et al., 2013; Chivulescu *et al*., 2018). Area is characterized by great compositional and structural variability (Cagliero *et al*., 2021). Also, mixed forests in beech (*Fagus sylvatica* L.) dominated sites are incredibly rare and serve for observation of natural processes in temperate zone (Curovic *et al*., 2020). Changes in this kind of forests are still present and trees with lowest diameter have significantly shorter time to passage (Govedar *et al*., 2021).

Forest pathogens occur in all categories of world's forests and all tree species are susceptible to pathogens in some degree (Tainter and Baker, 1996; Sinclair and Lyon, 2005; Edmonds, 2013). There are multiple drivers influencing trees susceptibility to forest pathogens including invasion by alien pathogens, climate change, emergence of more virulent strains of pathogen, hybridization of fungal species, latent of cryptic pathogens, establishment of new associations

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between vectors and pathogens and introduction of new forest practices (Ghelardini *et al*., 2016).

The first step in disease management includes detection, survey and monitoring (Edmonds, 2013). New diseases in European forests were reported at an increasing rate over the last century (Stenlid *et al*., 2011). One of such diseases is Dutch elm disease characterized with rapid spread and multiple epidemics in the world (Brasier, 2000). Dutch elm disease is common name for disease caused by group of species from genus *Ophiostoma* (Kirisits, 2013). This disease caused significant loss in elm populations, primarily leading to decrease of their economic importance (Karadžić, 2010; Kirsits, 2013). Survival of elm species due to this pathogen isn't completely endangered because trees need to be in certain stage of development prior to infection (Brasier, 1996). However, there is also increased occurrence of pathogens in domestic forests including impacts of wood decay fungi (Radulović *et al*., 2020; Karadžić *et al*., 2020; Radulović *et al*., 2021) and unusual transmission from different host species (Vemić and Radulović, 2021). This holds great ecological pressure on elm trees in domestic forests. Considering still active epidemic of Dutch elm disease in Europe and reducing the number of vitally trees, keystones for protection are finding and production of genetically resistant trees (Santini *et al*., 2010). Resources of this material should be also searched in untouched forests.

During the monitoring of trees health condition in "Biogradska Gora" National Park in Montenegro a symptoms of Dutch elm diseases on wych elm (*Ulmus glabra* Huds.) were recorded. Laboratory analyses confirmed presence of pathogen *Ophiostoma novo-ulmi* Brasier in symptomatic trees. This is the first report of *Ophiostoma novo-ulmi* in Biogradska Gora (Montenegro) as area previously considered to be without Dutch elm disease. These results will contribute to knowledge about distribution of *Ophiostoma novo-ulmi* and ways of using untouched forests for genetically resistant material.

MATERIAL AND METHODS

Locality of research

Symptoms of Dutch elm disease were noticed in summer of 2017 in locality of National Park "Biogradska Gora" (42º53′39" N, 19º36′15" E) after detailed examination of entire area. Twigs from symptomatic trees were collected for laboratory analyses.

Laboratory methods

Fungus was isolated using standard procedures described in Brasier (1981). After the surface sterilization of elm wood, a small pieces of dark xylem tissues were placed on selective media. Fresh grown pieces of mycelium were transferred on 3% MEA to obtain pure cultures. Storage of cultures was according recommendations described in Brasier (1981). Fresh 10 days old cultures were used for morphological and molecular identification.

Fungus was identified on both morphological and molecular criteria. Morphological identification was performed based on the characteristics of pure cultures and microstructures according to data described in Brasier (1981) and Brasier (1991). Examination of microstructures was performed using microscope AM Scope B120 C E1. Molecular identification was performed with Phire Plant Direct PCR Kit using ITS1/ITS4 combination of primers. Complete PCR procedure was performed according to manufacturer's recommendations.

RESULTS AND DISCUSSION

Recorded symptoms

Symptoms recorded in investigated area included wilting of trees and premature leaves shedding (Figure 1 A-B). Bark damages caused by the smaller European elm bark beetle (*Scolytus multistriatus* March.) provided additional evidence about presence of Dutch elm disease (Figure 1 C).

All symptoms were visible in summer period and based on their degree infection probably started some time earlier (Figure 1). Smaller twigs were equally infested as larger ones and fungus was more easily isolated from these twigs (Figure 1).

Figure 1. Symptoms of Dutch elm disease on wych elm (*Ulmus glabra*) A-B wilting of trees, C – damages of the smaller European elm bark beetle *Scolytus multistriatus*

Also, symptoms were recorded in less protected stands and between less protected stands and old-growth forest (Figure 1). Distant parts of old-growth forests were still without any symptoms of Dutch elm disease.

Identification of fungus

Species isolated from sampled tissues was identified as *Ophiostoma novo-ulmi* (Figure 2). Colonies were white, concentric with presence of aerial mycelium in central part (Figure 2 - A).

Conidia were from *Sporotrix* stage and abundant, single celled, holoblastic, ellipsoid to ovoid (Figure 2 - B). Also, some conidia aggregated in spore drops (Figure 2 B). Anamorph with synemates – *Pesotim* (*Graphium*) was absent in obtained cultures.

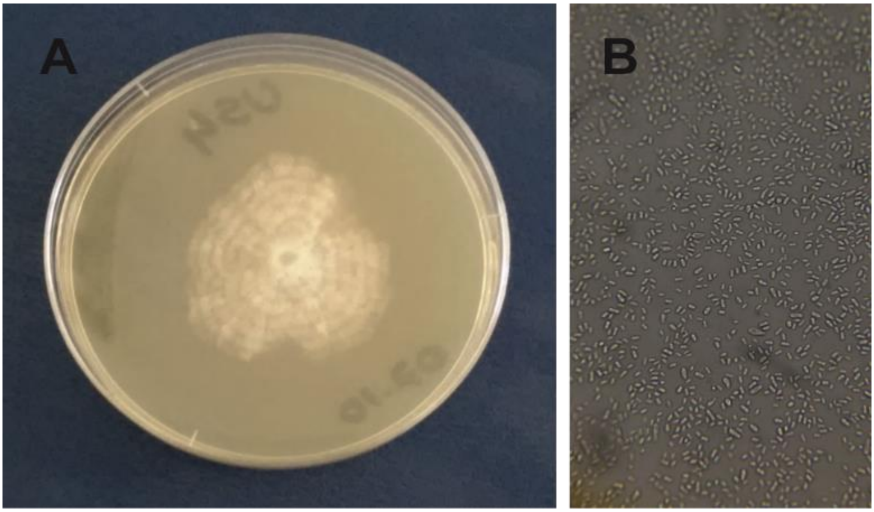

Figure 2. *Ophiostoma novo-ulmi:* A – characteristics of culture, B – conidial spores

Prior to this study "Biogradska Gora" National Park was considered to be free of Dutch elm disease. This is the first report of Dutch elm disease caused by *Ophiostoma novo-ulmi* in "Biogradska Gora" National Park. Results will have practical application in evaluating the potential of old-growth forests as source of genetically divergent trees that can be used in breeding programs for resistance to Dutch elm disease. Also, another practical application is for improving protection strategies of elm trees in "Biogradska Gora" National Park.

Dutch elm disease is caused by three closely related species from genus *Ophiostoma* including *Ophiostoma ulmi* (Buisman) Nannf., *Ophiostoma novo-ulmi* Braiser and *Ophiostoma himal-ulmi* Braiser & Mehrotra (Kirisits, 2013). From these species *Ophiostoma ulmi* was responsible for the first outbreak of disease in the Europe and North America on the beginning of 20th century (Gibbs, 1978; Brasier, 2000) and later in the middle of 20th century was replaced by *Ophiostoma novo-ulmi* (Brasier, 1991; Brasier, 2000). On the other side *Ophiostoma himal-ulmi* occurs endemically on Himalyan elm (*Ulmus wallichiana* (Planch.)) and currently there isn't reported its significant pathogenicity (Kirisits, 2013).

Species *Ophiostoma novo-ulmi* is considered to contain two subspecies whereby susp. novo-ulmi caused epidemic in Europe and subsp. americana caused epidemic in North America (Brasier, 1979; Brasier, 1991). From especially concerns is that hybridization between these subspecies exists and hybrid showed very rapid emergence and that further complex hybrids are now expanding (Brasier and Kirk, 2010). At this point more molecular analyses are required to evaluate routes of introduction in this area and characterization of present strain.

Fungus *Ophiostoma novo-ulmi* is still actively invasive and isolates decade after the outbreak showed similar characteristics as isolated early after the outbreak (Brasier and Webber, 2019). Anyway, clonal researches against DED are now more promising, despite that wych elm (*Ulmus glabra*) was more susceptible than the field elm (*Ulmus minor* Miller) (Solla *et al*., 2005). However, investigation of less genetically divergent *Ophiostoma novo-ulmi* populations showed that wych elm (*Ulmus glabra*) was less infested than the field elm (*Ulmus minor*) (Łakomy *et al.*, 2016). All this points to further researches about genetic background of booth pathogen and host in order to create proper strategies for preservation of this species.

National Park "Biogradska Gora" was well known for a long time as natural reserve where Dutch elm disease is relatively little or non-present. Previous studies (Karadžić, 1996; Karadžić *et al*., 1999) didn’t confirm presence of *Ophiostoma* species in this area pointing to conclusions that wych elm (*Ulmus glabra*) could be more resistant or "disease escape" is more likely in this kind of ecosystem. However, old-growth forests are much more resistant and tolerant to diseases in comparison to the other categories of forests (Lazarev, 2001). This means that sources of tolerant trees could still be found in these forests including Biogradska Gora especially because healthy trees were still found in this area. Based on these results tolerant trees and "disease escape" were more likely in distant parts of old-growth forest. Further protection of this National Park against DED should be in eliminating symptomatic trees in less protected area as well as adopting new strategies in regulating rules for the passage of visitors in more protected area – old-growth forest. This will reduce accidental transfer of inoculum or disturbing ecological stability of these forests.

CONCLUSIONS

Performed research leaded to next conclusions:

- ✓Fungus *Ophiostoma novo-ulmi* was isolated from wych elm (*Ulmus glabra*) trees in "Biogradska Gora" National Park.
- ✓All wych elm (*Ulmus glabra*) trees from whom *Ophiostoma novo-ulmi* was isolated had typical symptoms of Dutch elm disease without showing any form of tolerance.
- ✓All reported cases of *Ophiostoma novo-ulmi* in this study were in less protected zone and beginning of the old-growth forest located around lake "Biogradsko jezero".
- ✓Elm trees deep in old-growth forest didn’t have any symptoms of Dutch elm disease at the moment this study was performed.
- ✓This is the first report of Dutch elm disease in "Biogradska Gora" National Park.

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REFERENCES

Brasier, C.M. (1979): Dual origin of recent Dutch elm disease outbreaks in Europe. Nature, 281: 78-79. 10.1038/281078a0

Brasier, C.M. (1981): Laboratory investigation of *Ceratocystis ulmi*. In: R.J. Stipes & R.J. Campana eds. *Compendium of Elm Diseases*, pp. 76-79. St. Paul, MN, USA, APS Press. 96 pp.

Brasier, C.M. (1991): *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. Mycopathologia, 115(3): 151-161. 10.1007/BF00462219

Brasier, C.M. (1996): New horizons in Dutch elm disease control. In: *Reports on Forest Research 1996*. Edinburg, UK, Forestry commission, pp. 20-28.

Brasier, C.M. (2000): Intercontinental spread and continuing evaluation of the Dutch elm disease pathogens, In: C.P. Dunn ed. *The Elms: Breathing, Conservation and Disease Management*, pp. 61-62. Dordrecht, The Netherlands, Kluwer Academic Publishers. 361 pp. DOI:10.1007/978-1-4615-4507-1_4

Brasier, C.M. & Kirk S.A. (2010): Rapid emergence of hybrids between the two subspecies of *Ophiostoma novo-ulmi* with a high level of pathogenic fitness. Plant Pathology, 59(1): 186-199. https://doi.org/10.1111/j.1365-3059.2009.02157.x

Brasier, C.M., & Webber, J.F. (2019): Is there evidence for post-epidemic attenuation in the Dutch elm disease pathogen *Ophiostoma novo-ulmi*?. Plant Pathology, 68(5): 921-929. DOI:10.1111/ppa.13022

Cagliero, E., Morresi, D., Paradis, L., Curovic, M., Spalevic, V., Marchi, N., Meloni, F., Bentaleb, I., Motta, R., Garbarino, M., Lingua, E., Finsinger, W. (2021): Legacies of past human activities on one of the largest old-growth forests in the south-east European mountains. Vegetation History and Archaeobotany (2021). https://doi.org/10.1007/s00334-021-00862-x

Chivulescu, S., Leca, S., Silaghi, D., Cristea, V. (2018): Structural biodiversity and dead wood in virgin forests from Eastern Carpathians. Agriculture and Forestry, 64 (1): 177-188.

Curovic, M., Spalevic, V., Sestras, P., Motta, R., Dan, C., Garbarino, M., Vitali, A., Urbinati, C. (2020): Structural and ecological characteristics of mixed broadleaved old-growth forest (Biogradska Gora - Montenegro). Turkish journal of Agriculture and Forestry, 44: 428-438. DOI: 10.3906/tar-2003-103

Edmonds, R.L. (2013): General Strategies of Forest Disease Management, In: P. Gonthier & G. Nicolotti eds. *Infectious Forest Diseases*, pp. 29-49. London, UK, CAB International. 641 pp. https://doi.org/10.1079/9781780640402.0029

Ghelardini, L., Pepori, A.L., Luchi, N., Capretti, P., Santini, A. (2016): Drivers of emerging fungal diseases of forest trees. Forest Ecology and Management, 381: 235-246. https://doi.org/10.1016/j.foreco.2016.09.032

Gibbs, J.N. (1978): Intercontinental epidemiology of Dutch elm disease. Annual Review of Phytopathology, 16: 287-307. https://doi.org/10.1146/annurev.py.16.090178.001443

Govedar, Z., Kanjevac, B., Velkovski, N. (2021): Diameter structure stand and time of passage trees: An analysis in primeval forests Janj and Lom in Republic of Srpska (Bosnia and Herzegovina). Agriculture and Forestry, 67 (4): 89-102. doi:10.17707/AgricultForest.67.4.09

Karadžić, D. (1996): Proučavanje parazitske i saprofitske mikoflore u šumama Nacionalnog parka "Biogradska gora", private publisher. [in Serbian]

Karadžić, D. (2010): Šumska fitopatologija, Beograd, Srbija, Univerzitet u Beogradu, Šumarski fakultet. 774 pp. [in Serbian]

Karadžić, D., Knežević, M., Marković, D., Milijašević, T., Milenković, M. & Zarubica, B. (1999): Program zaštite i razvoja Nacionalnog parka „Biogradska gora" (segment šume), private publisher. [in Serbian]

Karadžić, D., Radulović, Z., Milenković, I., Miletić, Z. (2020): *Fomitopsis pinicola* (Fr.) P. Karst. and *Laetiporus sulphureus* (Fr.) Murrill Bioecological characteristics, significance and medicinal properties. Šumarstvo, 3-4: 29-50.

Kirisits, T. (2013): Dutch Elm Disease and Other *Ophiostoma* Diseases, In: P. Gonthier & G. Nicolotti eds. *Infectious Forest Diseases,* pp. 256-282. London, UK, CAB International. 641 pp. https://doi.org/10.1079/9781780640402.0256

Łakomy, A., Kwaśna, H., Kuźmiński, R., Napierała-Filipiak, A., Filipiak, M., Behnke, K., Behnke-Borowczyk, J. (2016): Investigation of *Ophiostoma* population infected elms in Poland. Dendrobiology, 76: 137-144.

Lazarev, V. (2001): Zdravstveno stanje prašuma Janj i Lom, U (grupa autora): Prašume "Janj" i "Lom", studija. Banja Luka, str. 117-128. [in Serbian]

Nagel, T., Zenner, E., Brang, P. (2013): Research in old-growth forests and forest reserves: implications for integrated forest management. In: D. Kraus & F. Krumm eds. *Integrative approaches as an opportunity for the conservation of forest biodiversity*, pp. 44-50. Joensuu, Finland, European Forest Institute, 284 pp.

Radulović, Z., Karadžić, D., Milenković, I., Stanivuković, Z. (2020): *Fomes fomentarius* (L.: Fr.) Fr. – Bioecological characteristics, economic importance and possibility of use for medical purposes (medicinal properties). Šumarstvo, 1-2: 13-31.

Radulović, Z., Karadžić, D., Milenković, I. (2021): The most common *Armillaria* in our forests and their medicinal properties. Šumarstvo 1-2: 25-48.

Santini, A., Pecori, F., Pepori, A.F., Ferrini, F., Ghelardini, L. (2010): Genotype × environment interaction and growth stability of several elm clones resistant to Dutch elm disease. Forest Ecology and Management, 260(6): 1017-1025. https://doi.org/10.1016/j.foreco.2010.06.025

Sinclair, W. & Lyon, H. (2005): Diseases of trees and shrubs, second edition, Ithaca, New York, Cornell University Press. 680 pp.

Solla, A., Bohnens, J., Collin, E., Diamandis, S., Franke, A., Gil, L., Burón, M., Santini, A., Mittempergher, L., Pinon, J., Vanden Broeck, A. (2005): Screening European Elms for Resistance to *Ophiostoma novo-ulmi*. Forest Science, 51(2): 134-141.

Stenlid, J., Oliva, J., Boberg, J.B. & Hopkins, A.J.M. (2011): Emerging Diseases in European Forest Ecosystems and Responses in Society. Forests, 2(2): 486-504. https://doi.org/10.3390/f2020486

Tainter, F.H. & Baker, F.A. (1996): Principles of forest pathology, New York, USA, Willey. 832 pp.

Vemić, A., Radulović, Z. (2021): Ocurrence of *Rutstroemia echinophila* (Bull.) Höhn. on beech cupules in Montenegro. Šumarstvo 3-4: 103-109.

Wirebuana, P., Y., A., P., Sadono, R., Matatula, J. (2022): Competition influences tree dimension, biomass distribution, and leaf area index of Eucalyptus urophylla in dryland ecosystems at East Nusa Tenggara. Agriculture and Forestry, 68 (1): 191-206. doi:10.17707/AgricultForest.68.1.12

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COMPETITION INFLUENCES TREE DIMENSION, BIOMASS DISTRIBUTION, AND LEAF AREA INDEX OF *EUCALYPTUS UROPHYLLA* IN DRYLAND ECOSYSTEMS AT EAST NUSA TENGGARA

SUMMARY

Competition is one of the important factors that determine the productivity of forest stands. However, the effect of competition on the growth dynamics at the individual tree level is rarely documented. This study investigated the influence of competition on the tree dimension, biomass, distribution, and leaf area index (LAI) of *Eucalyptus urophylla* established in dryland ecosystems in East Nusa Tenggara. Data collection was conducted by N-tree sampling method with the number of center points reaching 36 units. The competition among trees was quantified using Hegyi index. Several parameters were measured to describe tree characteristics, including diameter, height, aboveground biomass, and LAI. The results demonstrated a significant influence of competition on the tree dimension, biomass distribution, and LAI of *E. urophylla*. The individual tree performance declined along with the increase in competition. By contrast, the increased competition gradually improved the relative contribution of branch biomass to the total aboveground biomass. The exponential model best described the linkage between competition and the tree characteristics of *E. urophylla* in the study site. Referring to these findings, this study concluded that the growth dynamics of *E. urophylla* at the individual tree level in dryland ecosystems is substantially influenced by competition.

Keywords: Growth Dynamics, Hegyi index, N-trees Sampling, *Eucalyptus urophylla*, Leaf Area Index

INTRODUCTION

Understanding the growth dynamics at the individual tree level is principally required to optimize the activity of stand management, particularly in

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commercial plantation forests (Cao, 2014). Every tree has a different growth performance depending on its adaptability to the environmental condition, mainly for obtaining resources, such as light, water, and nutrients (Craine and Dybzinski, 2013). However, the availability of resources in forest ecosystems is relatively limited (Álvarez-Yépiz *et al.*, 2017). Thus, trees should compete with each other to derive adequate resources for supporting their growth process (Lamonica *et al.*, 2020). Trees having good adaptability will show superior performance, whereas weak trees will die naturally or exhibit inferior growth (Rozendaal *et al.*, 2020). This condition directly confirms that the distributions of tree dimensions at the stand level are unequal although they were established at a similar time period. The information about tree growth variation is generally used as a basic consideration to determine the best silviculture prescriptions for improving the productivity of plantation forests.

The occurrence of competition in forest ecosystems is a natural mechanism that plays an essential role in biogeochemical cycle (Andrés, 2019). A study reported that an intense competition will construct a high dense canopy (Looney *et al.*, 2016) and potentially increase the ability of forests for carbon absorption (Farrior *et al.*, 2013). A dense canopy also provides an important contribution to creating a micro-climate condition under the forest stand (Arx *et al.*, 2013). Another study reported that a dense canopy has the potential to minimize rainfall kinetic energy into a forest floor that causes run-off and erosion (Li *et al.*, 2019). The competition among trees is also one of the primary factors that influence the litter quantity in forest ecosystems wherein a high competition improves the rate of canopy litterfall (Silver *et al.*, 2014). This condition indicates an indirect linkage between tree competition and nutrient dynamics in forests.

Despite providing ecological benefits, the occurrence of competition among trees should be controlled intensively because it has a negative effect on forest productivity, which is primarily related to the economic aspect (Forrester *et al.*, 2013). A high competition substantially increases the mortality rate at the stand level (Das *et al.*, 2011, Ruiz-Benito *et al.*, 2013, Wang *et al.*, 2017, Maringer *et al.*, 2021). A high competition also decreases the production of merchantable wood from forest ecosystems (Bembenek *et al.*, 2014). In addition, an increased intense competition has the potential to inhibit stand growth due to the low availability of resources for trees (Yang *et al.*, 2019). Therefore, most forest managers commonly undertake thinning and pruning activities to reduce the competition level (Keyser and Zarnoch, 2012; Nogueira *et al.*, 2015; Filho *et al.*, 2018). These explanations demonstrate that the availability of information about competition is exceptionally required to support the implementation of sustainable forest management, particularly in plantation forests.

Several references explain that the study of competition in forest ecosystems is immensely specific given that every tree species has a variety of tolerance to competition (Kunstler *et al.*, 2012, Barabás *et al.*, 2016, Fichtner *et al.*, 2017). Moreover, the level of competition in forests is influenced by site quality and intensity of maintenance (Moreno-Fernández *et al.*, 2014, Zhang *et* *al.*, 2016). In another word, the different types of forest and management strategies will result in different competition rates. This study investigated the influence of competition on individual tree performance of *Eucalyptus urophylla* S.T. Blake established in dryland ecosystems in East Nusa Tenggara. As one of the fast-growing species, *E. urophylla* is a native species from Indonesia and is commercially developed in several countries, such as Vietnam and China. However, the study on the competition of *E. urophylla* is rarely documented, particularly from Indonesia. This study aimed to assess the influence of nearest-neighbor competition on the tree dimensions, biomass distribution, and leaf area index (LAI) of *E. urophylla*.

MATERIAL AND METHODS

The study location is situated in an *E. urophylla* plantation which is developed for dryland ecosystems in East Nusa Tenggara. This area is located in Timor Tengah Selatan District, around 180 km at the northeastern Kupang, the capital city of East Nusa Tenggara Provinces. The geographic coordinates of the research site are in S9°50′0″ to S9°50′15″ and E124°15′30″ to E124°16′0″ (Figure 1). The total area of *E. urophylla* plantation in this location is 25 ha and managed by Timor Tengah Selatan Forest Management Unit. In the initial periods, before being converted into an *E. urophylla* plantation, the vegetation cover in this area was dominated by Imperata cylindrica (Sadono *et al.*, 2020). In 1997, the local government conducted reforestation using *E. urophylla* as the primary species for rehabilitation. In addition to having a rapid growth, *E. urophylla* is a native species from this location (Almulqu *et al.*, 2019). Therefore, the implementation of reforestation is also expected to support conservation for indigenous species from East Nusa Tenggara.

The study site has an altitude of 800 m above sea level. Topography is dominated by a hilly area with a slope level of 15%. The soil type is classified as cambisol with a high cation exchange capacity and rich phosphorus content (Table 1). This location is categorized into a humid condition with an average air humidity of around 85.5%. The mean daily temperature is 29°C. Annual rainfall ranges from 1,300 mm year^{-1} to 1,800 mm year^{-1} during the past five years from 2016 to 2020. The highest rainfall occurs in January. The study site has dry periods for 7 months from March to September.

Data collection was undertaken by a field inventory using N-trees sampling method. In this sampling technique, the process of tree measurement was conducted at the nearest trees from the center point (Mirzaei and Eslam, 2016). The principle of this method is similar to the point-centered quarter method, which is frequently used in ecological surveys, primarily for tree vegetation (Haxtema *et al.*, 2012).

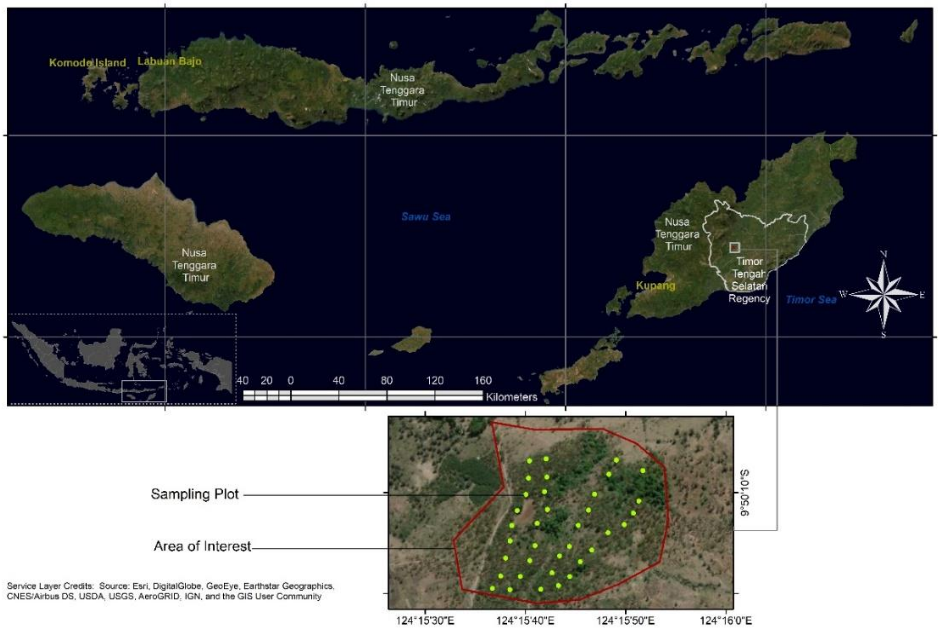

Figure 1. Study location of *E. urophylla* plantation managed by Timor Tengah Selatan Forest Management Unit. The red polygon indicates the border compartment of *E. urophylla* stand, which is allocated as a limited production forest. The green point signifies the coordinate of sampling plots quantified from the subject tree

Table 1. Soil characteristics of the study location based on the acidity level, soil organic carbon, total nitrogen, available phosphorus, exchangeable potassium, and cation exchange capacity

Soil parameter	*Symbol*	*Units*	*Value*
Soil acidity	pH H_2O	-	6.24±0.59
Soil organic carbon	SOC	%	0.84±0.35
Total nitrogen	TN	%	0.59±0.39
Available phosphorus	Av-P	ppm	41.79±0.68
Exchangeable potassium	Exc-K	cmolc(+) kg^{-1}	0.70±0.46
Cation exchange capacity	CEC	cmolc(+) kg^{-1}	34.51±0.59

However, the numbers of sample trees from both methods were considerably different. The use of N-tree sampling method involved six sample trees, whereas the use of point-centered quarter method measured four sample trees (Silva *et al.*, 2017).

In this study, the main subject tree was positioned as the center point, whereas the competing trees were assumed as the nearest-neighbor trees (Figure 2). The total subject trees observed in this study were 36 trees with an average distance of 50 m between subject trees.

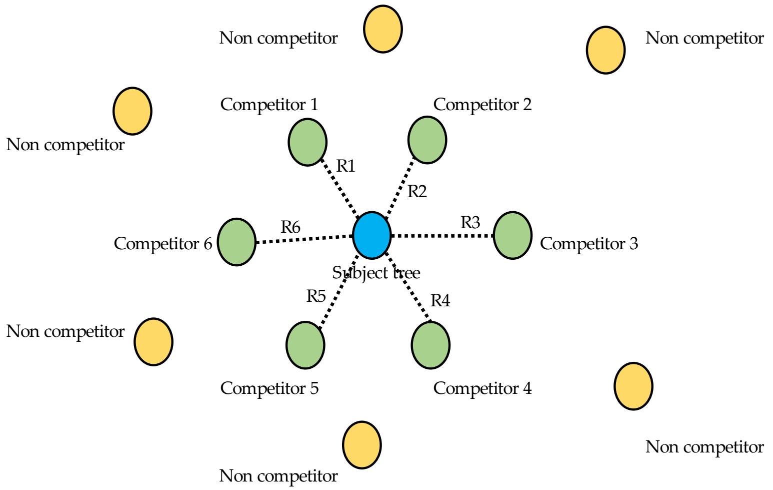

Figure 2. Illustration of N-tree sampling method for the quantification of tree characteristics and competition among trees in the *E. urophylla* plantation. The blue circle indicates the subject tree as the main focus of research, whereas the green circles show the competing trees. The yellow circles show the non-competitor trees, and R_n represents the distance between the subject tree and competing trees

Several parameters were quantified from each subject tree, i.e., diameter, height, aboveground biomass, and LAI. For competitor trees, the parameter measurement was focused on diameter and distance between each competing tree with the subject tree. The diameter of every tree was measured using a diameter tape at 1.3 m from aboveground. The distance between the subject tree and competing trees was quantified by a laser distance meter. The tree height was estimated from the aboveground to the top crown by a Haglof vertex. The biomass distribution from every subject tree was estimated at aboveground in each component (stem, branch, and foliage) using allometric equations (Table 2). Meanwhile, the LAI was quantified by hemispherical photography. To facilitate this stage, we obtained the crown photograph using the Nikon Camera with a fish-eye lens (FC-08).

For every subject tree, the collection of crown image was conducted at four different positions (Figure 3) (Rody *et al.*, 2014). This measurement was implemented to increase the accuracy of crown image analysis, which was processed by Hemisfer software version 3.1. To obtain a more stable image, we supported the camera by a tripod set up at 1 m from the aboveground (Hakamada *et al.*, 2016). All images were analyzed alternately. Then, LAI of subject tree was determined based on the mean value of LAI from four photographs.

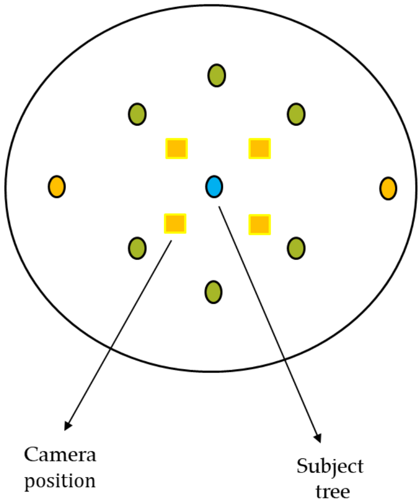

Figure 3. Camera positions for capturing the crown image of every subject tree. The blue circle is a subject tree, whereas the yellow square indicates the camera position. The green circle shows the competing trees, there the orange circle represents the non-competitor trees.

Table 2. Allometric equations for estimating biomass accumulation in the stem, branch, and foliage of *E. urophylla* in dryland ecosystems in East Nusa Tenggara. The symbol "Y" indicates the sum of biomass in the tree component, whereas "D" represents the tree diameter as a predictor variable

Tree component	*Equations*	R^2	*AIC*	*RMSE*
Stem	Y = 0.035 + 0.008D	0.95	−135.27	0.014
Branch	Y = −0.016 + 0.003D	0.44	−121.83	0.021
Foliage	Y = 0.0002 + 0.001D	0.85	−201.74	0.002

Source: Almulqu et al. (2019)

In this study, the competition between the subject tree and competitor trees was estimated by Hegyi index (McTague and Weiskittel, 2016). This parameter is commonly used to calculate the competition level among trees in various forest ecosystems (Ledermann and Stage, 2001, Das *et al.*, 2011, Fraver *et al.*, 2014, Kang *et al.*, 2017). The Hegyi index was selected to quantify the competition given that it is simpler than the other parameters, wherein the competition between the subject tree and competitor trees was calculated based on their diameter and distance (Maleki *et al.*, 2015). The mathematical formula for Hegyi index is expressed below:

$$CI_H = \sum_{j=1}^{n}\left[\frac{\left(\frac{d_j}{d_i}\right)}{R_{ij}}\right] \quad (1)$$

where CI_H is the Hegyi index, d_j indicates the diameter of competitor tree *j* (cm), d_i represents the diameter of subject tree *i* (cm), R_{ij} shows the distance between the subject tree *i* and competitor tree *j* (m), and n refers to the number of trees included in the quantification process (excluding subject tree). Then, the outcomes of Hegyi index were categorized into three classes to describe the competition level, namely, low (CI_H < 0.5), moderate (0.5 ≤ CI_H ≤ 1.0), and high (1.0 > CI_H).

Statistical analysis was applied with a significant level of 5%. A descriptive test was conducted to identify the data attributes, including the minimum, maximum, mean, and standard deviation. The normality of data was tested using Shapiro–Wilk test. The homogeneity of variance among competition level was evaluated by Bartlett's test. Comparison averages of individual tree dimension, aboveground biomass, and LAI in *E. urophylla* among the competition classes were examined using analysis of variance (ANOVA) and followed by honestly significant difference (HSD) Tukey's. Moreover, the analysis of regression was applied to identify the relationship between competition and individual tree characteristics. Three different models were tested in this stage, namely, linear, power, and exponential. The detailed equation for every model is demonstrated below:

$$Y = a + bX \tag{2}$$

$$Y = aX^b \tag{3}$$

$$Y = ae^{bX} \tag{4}$$

where Y indicates the individual tree characteristics (diameter, height, aboveground biomass, and LAI), a and b are the fitted parameters, e demonstrates an Euler's number (2.71828), and X shows the Hegyi index. Several indicators were used to determine the best model, i.e., the significant result of ANOVA test, the significant result of fitted parameters test (a and b), adjusted R squared (R^2 adj), residual standard error (RSE), akaike information criterion (AIC), mean absolute bias (MAB), and normalized root mean square error (NRMSE).

The indicators of ANOVA test in fitted parameters test, namely, R^2 adj, RSE, and AIC, were used to examine the model fitting. Meanwhile, MAB and NRMSE were applied to assess the validation test. The equations for calculating those parameters are presented below:

$$R^2\,adj = 1 - \left[\frac{(1 - R^2)(n-1)}{n-k-1}\right] \tag{5}$$

$$RSE = \sqrt{\frac{1}{n-2}\sum_{i=1}^{n}\left(\Upsilon - \hat{\Upsilon}\right)^2} \tag{6}$$

$$AIC = n\log\left(\frac{RSS}{n}\right) + 2k + \frac{2k + (k+1)}{n-k-1} \tag{7}$$

$$MAB = \sum_{1}^{n}\frac{(\Upsilon - \widehat{\Upsilon})}{n} \tag{8}$$

$$NRMSE = \frac{RMSE}{\theta} \tag{9}$$

where Υ is the actual tree characteristics obtained from field inventory, $\widehat{\Upsilon}$ indicates the estimated tree parameters from the fitted model, n represents the sample size, k shows the number of parameters, R^2 is the coefficient of determination, RSS signifies the residual sum of squares from the fitted model, $RMSE$ is the root mean square error, and θ shows the mean observation value of the tree characteristics.

Given that the sample size was relatively small (36 trees), the validation test was conducted by the leave-one-out cross-validation method (LOOCV). LOOCV is frequently applied to regression analysis with a small sample size (Castillo-Santiago *et al.*, 2010, Altanzagas *et al.*, 2019, Tetemke *et al.*, 2019). The best model had to demonstrate the significant results of ANOVA and fitted parameter test, the highest R^2 adj, and the lowest RSE, AIC, MAB, and NRMSE.

RESULTS

The summarized observation results demonstrated that the competition level of *E. urophylla* in the study area relatively varied although these plants were developed in sites with similar qualities (Table 3). This study recorded that the majority of trees had a moderate competition level. The lowest value of Hegyi index obtained from the field survey was 0.39, whereas the highest value was 1.53. Compared with other competition levels, the total subject trees at the low competition level were considerably lower by approximately nine trees.

Table 3. Summary statistics of Hegyi index estimation in every competition level. Data are presented as the number of trees, minimum, maximum, mean, and standard deviation

Competition	CI_H	Number Of Tree	Min	Max	Mean	SD
Low	<0.5	9	0.39	0.49	0.43	0.03
Moderate	0.5-1.0	17	0.52	0.78	0.72	0.12
High	>1.0	10	1.08	1.53	1.27	0.15

Note: CI_H showed Hegyi index

The occurrence of competition indicates a significant effect on individual tree characteristics (Table 4). The high competition level substantially declined tree dimension, biomass accumulation, and LAI of *E. urophylla* in dryland ecosystems. A similar trend was also observed with the relative contribution of stem and foliage biomass to total aboveground biomass (Figure 4). By contrast, this study discovered that the relative contribution of branch biomass to the total aboveground biomass significantly improved along with the increase in the competition level.

Table 4. Comparison of the means of the individual tree characteristics in each competition class. Data are presented in diameter, height, biomass in each component, total aboveground biomass, and LAI

Parameter	*Competition level*			*P*
	Low	*Moderate*	*High*	
D (cm)	43.87 ± 9.84a	40.68 ± 6.45a	25.66 ± 5.00b	<0.001**
H (m)	40.94 ± 6.31a	37.86 ± 7.32a	23.55 ± 5.25b	<0.001**
SB (Mg)	1.46 ± 0.91a	1.15 ± 0.46a	0.35 ± 0.19b	<0.001**
BB (Mg)	0.29 ± 0.15a	0.23 ± 0.08a	0.08 ± 0.04b	<0.001**
FB (Mg)	0.04 ± 0.02a	0.04 ± 0.01a	0.01 ± 0.01b	<0.001**
AGB (Mg)	1.81 ± 1.43a	1.43 ± 0.56a	0.45 ± 0.25b	<0.001**
LAI	1.19 ± 0.20a	1.13 ± 0.15a	0.70 ± 0.17b	<0.001**

Note: D (diameter at breast height); H (tree height); SB (stem biomass); BB (branch biomass); FB (foliage biomass); AGB (total aboveground biomass); LAI (leaf area index)

Although they differed significantly, our study realized that the individual tree characteristics of *E. urophylla* at the low and moderate competition levels were statistically similar (Table 4). These findings were also followed by the relative contribution of every tree component to the total aboveground biomass (Figure 4).

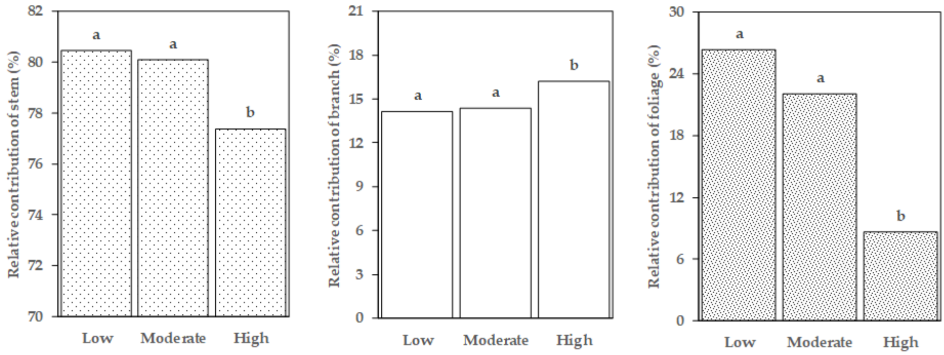

Figure 4. Comparison means of the relative contribution of biomass in every component (stem, branch, and foliage) to the total aboveground biomass of *E. urophylla* at the different competition levels; A similar letter above graph showed no significant different according to the honestly significant difference (HSD) Tukey's

Table 5. Parameter estimates and model evaluation statistics of every equation for describing the relationship between the competition and individual tree characteristics of *E. urophylla* in dryland ecosystems in East Nusa Tenggara

Parameters	*Equations*	*a*	*b*	*R^2 adj*	*RSE*	*AIC*	*MAB*	*NRMSE*
D	$Y = a + bX$	57.480	−25.402	0.561	7.449	175.266	0.131	0.160
	$Y = aX^b$	29.740	−0.570	0.599	0.197	−6.488	0.045	0.053
	$Y = ae^{bX}$	**64.033**	**−0.738**	**0.626**	**0.190**	**−8.255**	**0.041**	**0.048**
H	$Y = a + bX$	54.191	−24.443	0.571	7.029	172.366	0.114	0.005
	$Y = aX^b$	27.356	-0.597	0.584	0.212	−2.671	0.045	0.053
	$Y= ae^{bX}$	**61.363**	**−0.778**	**0.620**	**0.203**	**−4.948**	**0.034**	**0.041**
AGB	$Y = a + bX$	3.298	−2.233	0.408	0.880	68.461	0.409	0.041
	$Y = aX^b$	0.726	−1.457	0.599	0.502	40.431	2.558	0.022
	$Y = ae^{bX}$	**5.150**	**−1.884**	**0.626**	**0.485**	**38.672**	**0.137**	**0.028**
LAI	$Y = a + bX$	1.568	−0.687	0.626	0.177	−11.803	0.204	0.227
	$Y = aX^b$	0.814	−0.578	0.596	0.200	−5.547	0.177	0.156
	$Y = ae^{bX}$	**1.779**	**−0.754**	**0.635**	**0.190**	**−8.083**	**0.115**	**0.209**

Note: D (diameter at breast height); H (tree height); AGB (total aboveground biomass); LAI (leaf area index); Bold equations indicate the best model for describing the relationship between competition and individual tree attributes; All models indicate the significance of ANOVA and fitted parameters.

Nevertheless, the growth performance of *E. urophylla* at a low competition level was higher than that at the moderate competition level by approximately 7%–22%. Meanwhile, the occurrence of high competition affected the loss of growth by around 16%–76%. At a high competition level, the relative contribution of branch biomass increased almost to 12.5% compared with the low and moderate competition levels.

The results demonstrated that every model provided a good fit to describe the linkage between the competition and tree characteristics of *E. urophylla* (Table 5). However, the use of exponential model indicated the best accuracy for explaining the effect of competition on individual tree dimension, biomass accumulation, and LAI in *E. urophylla*. This model can explain more than 60% of the growth variation in *E. urophylla*, which affected by competition levels (Figure 5). Moreover, the exponential model showed the lowest RSE, AIC, MAB, and NRMSE. These findings imply that the exponential model is more reliable and valid than the linear and power models in describing the relationship between competition level and individual tree characteristics of *E. urophylla*. According to the best model, the high competition significantly declined the individual tree dimension, total aboveground biomass, and LAI of *E. urophylla* in dryland ecosystems at East Nusa Tenggara.

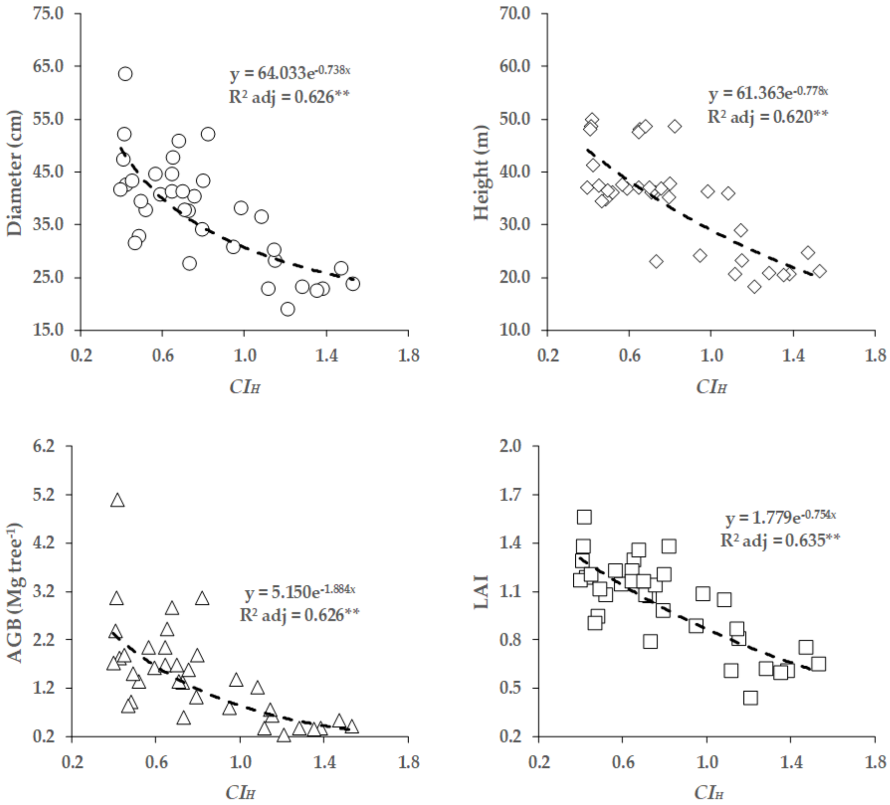

Figure 5. Scatter plots describing the pattern of relationship between competition and individual tree attributes, i.e. diameter, height, AGB (aboveground biomass), and LAI (leaf area index).

DISCUSSION

Competition among trees is a natural process that commonly in every type of forest ecosystem due to the limited availability of resources for supplying trees requirement. In general, two classifications of competition, including interspecific and intraspecific, transpire in forests (Skálová *et al.*, 2013). Interspecific competition occurs between individual trees from different species (Barabás *et al.*, 2016). This competition can be found in natural or mixed forests with multiple tree species. Meanwhile, intraspecific competition refers to the competition between individual trees from the same species (Yang *et al.*, 2019). This type of competition can be discovered in plantation forests with a monoculture species. The explanations showed that the primary focus of this study was to understand the intraspecific competition between E. urophylla trees developed in dryland ecosystems at East Nusa Tenggara.

The occurrence of intraspecific competition is generally more intense than interspecific competition (Saha *et al.*, 2014, Barabás *et al.*, 2016, Adler *et al.*, 2018, Yang *et al.*, 2019). Intraspecific competition normally occurs because individual trees have the same niche; as a result, trees are competing for exactly the same resources (Zhou *et al.*, 2018). This study showed that the level of competition between individual trees highly varied although they were planted at the same location. This circumstance can possibly occur given the gradient of biophysical characteristics in micro-site conditions. Our argument was also supported by the previous studies which reported a variety of soil fertilities in a compartment (Washitani and Tang, 1991, Gömöry *et al.*, 2011, Salekin *et al.*, 2019). Difference in soil fertility would provide the various resource supply for supporting tree growth. This statement was also emphasized by the description of the study site, in which the land configuration of the study location was dominated by hilly area. Thereby, the slope variation can influence the intensity of leaching and erosion in forest soil and cause the different soil fertilities in the site.

The growth performance of *E. urophylla* significantly declined along with the increased competition level. This finding was observed because at the high competition level, trees obtain smaller resources than at a lower competition (Forrester *et al.*, 2013). Consequently, the size of tree dimension, biomass production, and LAI were relatively lower than those of trees having low competition levels. Previous studies also reported similar outcomes to our findings, wherein a high competition considerably declined the growth performance of individual trees (Yan *et al.*, 2015, Resende *et al.*, 2018, Truax *et al.*, 2018). However, the increased competition improved the relative contribution of branch biomass to the total aboveground biomass, whereas a different trend was observed in stems and foliage. Survival during an intense competition is a natural mechanism of trees. As an explanation, the more intense the competition, a tree will expand its crown to obtain more light and space and to support this process, and branch development will occur more intensively than stems and foliage. This process explains why the relative contribution of branch biomass slightly increased along with the rising competition. However, a more intense competition accelerates the rate of canopy litterfall, thus stimulating natural pruning (Silva *et al.*, 2014). This condition confirms why the branch biomass significantly declined with the higher competition although a different pattern was recorded for its relative contribution to the total aboveground biomass.

CONCLUSIONS

This study concluded that the occurrence of competition significantly influences the individual tree characteristics of *E. urophylla* developed in dryland ecosystems in East Nusa Tenggara. A high competition substantially decreased the individual tree dimension, total aboveground biomass, and LAI of *E. urophylla*. The relative contribution of branch biomass to the total aboveground biomass significantly improved along with the increased competition level. The exponential model best described the relationship between the competition level and individual tree dimension.

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REFERENCES

Adler, P. B., Smull, D., Beard, K. H., Choi, R. T., Furniss, T., Kulmatiski, A., Meiners, J. M., Tredennick, A. T., & Veblen, K. E. (2018): Competition and coexistence in plant communities: intraspecific competition is stronger than interspecific competition. Ecology Letters, 21(9): 1319–1329. https://doi.org/10.1111/ele.13098

Almulqu, A. A., Arpornpong, N., & Boonyanuphap, J. (2019): Biomass estimation and allometric equation for tree species in dry forest of East Nusa Tenggara, Indonesia. Forestry Ideas, 25(2): 369–384.

Altanzagas, B., Luo, Y., Altansukh, B., Dorjsuren, C., Fang, J., & Hu, H. (2019): Allometric equations for estimating the above-ground biomass of five forest tree species in Khangai, Mongolia. Forests, 10(8): 1–17. https://doi.org/10.3390/f10080661

Álvarez-Yépiz, J. C., Búrquez, A., Martínez-Yrízar, A., Teece, M., Yépez, E. A., & Dovciak, M. (2017): Resource partitioning by evergreen and deciduous species in a tropical dry forest. Oecologia, 183(2): 607–618. https://doi.org/10.1007/s00442-016-3790-3

Andrés, E. G. (2019): Interactions between climate and nutrient cycles on forest response to global change: The role of mixed forests. Forests, 10(8): 1–26. https://doi.org/10.3390/f10080609

Arx, G., Graf Pannatier, E., Thimonier, A., & Rebetez, M. (2013): Microclimate in forests with varying leaf area index and soil moisture: Potential implications for seedling establishment in a changing climate. Journal of Ecology, 101(5): 1201–1213. https://doi.org/10.1111/1365-2745.12121

Barabás, G., Michalska-Smith, M. J., & Allesina, S. (2016): The effect of intra- and interspecific competition on coexistence in multispecies communities. American Naturalist, 188(1): 1–12. https://doi.org/10.1086/686901

Bembenek, M., Karaszewski, Z., Kondracki, K., Łacka, A., Mederski, P. S., Skorupski, M., Strzeliński, P., Sułkowski, S., & Węgiel, A. (2014): Value of merchantable timber in scots pine stands of different densities. Drewno, 57(192): 134–142. https://doi.org/10.12841/wood.1644-3985.S14.09

Cao, Q. V. (2014): Linking individual-tree and whole-stand models for forest growth and yield prediction. Forest Ecosystems, 1(1): 1–8. https://doi.org/10.1186/s40663-014-0018-z

Castillo-Santiago, M. A., Ricker, M., & De Jong, B. H. J. (2010): Estimation of tropical forest structure from spot-5 satellite images. International Journal of Remote Sensing, 31(10): 2767–2782. https://doi.org/10.1080/01431160903095460

Craine, J. M., & Dybzinski, R. (2013): Mechanisms of plant competition for nutrients, water and light. Functional Ecology, 27(4): 833–840. https://doi.org/10.1111/1365-2435.12081

Das, A., Battles, J., Stephenson, N. L., & van Mantgem, P. J. (2011): The contribution of competition to tree mortality in old-growth coniferous forests. Forest Ecology and Management, 261(7): 1203–1213. https://doi.org/10.1016/j.foreco.2010.12.035

Farrior, C. E., Dybzinski, R., Levin, S. A., & Pacala, S. W. (2013): Competition for water and light in closed-canopy forests: A tractable model of carbon allocation with implications for carbon sinks. American Naturalist, 181(3): 314–330. https://doi.org/10.1086/669153

Fichtner, A., Härdtle, W., Li, Y., Bruelheide, H., Kunz, M., & von Oheimb, G. (2017): From competition to facilitation: how tree species respond to neighbourhood diversity. Ecology Letters, 20(7): 892–900. https://doi.org/10.1111/ele.12786

Filho, A. C. F., Mola-yudego, B., & González-, J. R. (2018): Thinning regimes and initial spacing for Eucalyptus plantations in Brazil. Anals Da Academia Brasileira de Ciencias, 90: 255–265.

Forrester, D. I., Wiedemann, J. C., Forrester, R. I., & Baker, T. G. (2013): Effects of planting density and site quality on mean tree size and total stand growth of Eucalyptus globulus plantations. Canadian Journal of Forest Research, 43: 846–851.

Fraver, S., D'Amato, A. W., Bradford, J. B., Jonsson, B. G., Jönsson, M., & Esseen, P. A. (2014): Tree growth and competition in an old-growth Picea abies forest of boreal Sweden: Influence of tree spatial patterning. Journal of Vegetation Science, 25(2): 374–385. https://doi.org/10.1111/jvs.12096

Gömöry, D., Paule, L., & Gömöryová, E. (2011): Effects of microsite variation on growth and adaptive traits in a beech provenance trial. Journal of Forest Science, 57(5): 192–199. https://doi.org/10.17221/88/2010-jfs

Hakamada, R., Giunti Neto, C., de Lemos, C. C. Z., Silva, S. R., Otto, M. S. G., Hall, K. B., & Stape, J. L. (2016): Validation of an efficient visual method for estimating leaf area index in clonal Eucalyptus plantations. Southern Forests, 78(4): 275–281. https://doi.org/10.2989/20702620.2016.1201641

Haxtema, Z., Temesgen, H., & Marquardt, T. (2012): Evaluation of n-Tree Distance Sampling for Inventory of Headwater Riparian Forests of Western Oregon. Western Journal of Applied Forestry, 27(3): 109–117. https://doi.org/10.5849/wjaf.10-035

Kang, D., Deng, J., Qin, X., Hao, F., Guo, S., Han, X., & Yang, G. (2017): Effect of competition on spatial patterns of oak forests on the Chinese Loess Plateau. Journal of Arid Land, 9(1): 122–131. https://doi.org/10.1007/s40333-016-0024-9

Keyser, T. L., & Zarnoch, S. J. (2012): Thinning, age, and site quality influence live tree carbon stocks in upland hardwood forests of the Southern Appalachians. Forest Science, 58(828): 407–418.

Kunstler, G., Lavergne, S., Courbaud, B., Thuiller, W., Vieilledent, G., Zimmermann, N. E., Kattge, J., & Coomes, D. A. (2012): Competitive interactions between forest trees are driven by species' trait hierarchy, not phylogenetic or functional similarity: Implications for forest community assembly. Ecology Letters, 15(8): 831–840. https://doi.org/10.1111/j.1461-0248.2012.01803.x

Lamonica, D., Pagel, J., Valdés-Correcher, E., Bert, D., Hampe, A., & Schurr, F. M. (2020): Tree potential growth varies more than competition among spontaneously established forest stands of pedunculate oak (*Quercus robur*). Annals of Forest Science, 77(3): 1–18. https://doi.org/10.1007/s13595-020-00981-x

Ledermann, T., & Stage, A. R. (2001): Effects of competitor spacing in individual-tree indices of competition. Canadian Journal of Forest Research, 31(12): 2143–2150. https://doi.org/10.1139/x01-153

Li, G., Wan, L., Cui, M., Wu, B., & Zhou, J. (2019): Influence of canopy interception and rainfall kinetic energy on soil erosion under forests. Forests, 10(6): 1–15. https://doi.org/10.3390/f10060509

Looney, C. E., D'Amato, A. W., Fraver, S., Palik, B. J., & Reinikainen, M. R. (2016): Examining the influences of tree-to-tree competition and climate on size-growth relationships in hydric, multi-aged *Fraxinus nigra* stands. Forest Ecology and Management, 375: 238–248. https://doi.org/10.1016/j.foreco.2016.05.050

Maleki, K., Kiviste, A., & Korjus, H. (2015): Analysis of individual tree competition effect on diameter growth of silver birch in Estonia. Forest Systems, 24(2): 1–14. https://doi.org/10.5424/fs/2015242-05742

Maringer, J., Stelzer, A. S., Paul, C., & Albrecht, A. T. (2021): Ninety-five years of observed disturbance-based tree mortality modeled with climate-sensitive accelerated failure time models. European Journal of Forest Research, 140(1): 255–272. https://doi.org/10.1007/s10342-020-01328-x

McTague, J. P., & Weiskittel, A. R. (2016): Individual-tree competition indices and improved compatibility with stand-level estimates of stem density and long-term production. Forests, 7(10): 1–16. https://doi.org/10.3390/f7100238

Mirzaei, M., & Eslam, A. (2016): Comparison of fixed area and distance sampling methods in open forests : case study of Zagros Forest, Iran. Journal of Forestry Research, 27(5): 1121–1126. https://doi.org/10.1007/s11676-016-0239-9

Moreno-Fernández, D., Sánchez-González, M., Álvarez-González, J. G., Hevia, A., Majada, J. P., Cañellas, I., & Gea-Izquierdo, G. (2014): Response to the interaction of thinning and pruning of pine species in Mediterranean mountains. European Journal of Forest Research, 133(5): 833–843. https://doi.org/10.1007/s10342-014-0800-z

Nogueira, G. S., Marshall, P. L., Leite, H. G., & Campos, J. C. C. (2015): Thinning Intensity and Pruning Impacts on Eucalyptus Plantations in Brazil . International Journal of Forestry Research, 2015: 1–10. https://doi.org/10.1155/2015/168390

Resende, R. T., Soares, A. A. V., Forrester, D. I., Marcatti, G. E., dos Santos, A. R., Takahashi, E. K., e Silva, F. F., Grattapaglia, D., Resende, M. D. V., & Leite, H. G. (2018): Environmental uniformity, site quality and tree competition interact to determine stand productivity of clonal Eucalyptus. Forest Ecology and Management, 410: 76–83. https://doi.org/10.1016/j.foreco.2017.12.038

Rody, P. Y., Ribeiro, A., Pezzopane, J. E. M., Gleriani, J. M., Almeida, A. Q., & Leite, F. P. (2014): Estimates of the leaf area index (LAI) using LAI-2000 and hemispherical photo in Eucalyptus plantation. Ciencia Florestal, 24(4): 925–934. https://doi.org/10.1590/1980-509820142404012

Rozendaal, D. M. A., Phillips, O. L., Lewis, S. L., Affum-Baffoe, K., Alvarez-Davila, E., Andrade, A., Aragão, L. E. O. C., Araujo-Murakami, A., Baker, T. R., Bánki, O., Brienen, R. J. W., Camargo, J. L. C., Comiskey, J. A., Djuikouo Kamdem, M. N., Fauset, S., Feldpausch, T. R., Killeen, T. J., Laurance, W. F., Laurance, S. G. W., … Vanderwel, M. C. (2020): Competition influences tree growth, but not mortality, across environmental gradients in Amazonia and tropical Africa. Ecology, 101(7): 1–11. https://doi.org/10.1002/ecy.3052

Ruiz-Benito, P., Lines, E. R., Gómez-Aparicio, L., Zavala, M. A., & Coomes, D. A. (2013): Patterns and Drivers of Tree Mortality in Iberian Forests: Climatic Effects Are Modified by Competition. PLoS ONE, 8(2): https://doi.org/10.1371/journal.pone.0056843

Sadono, R., Wardhana, W., Wirabuana, P. Y. A. P., & Idris, F. (2020): Productivity evaluation of *Eucalyptus urophylla* plantation established in dryland ecosystems, East Nusa Tenggara. J. Degrade. Min. Land Manage, 8(1): 2502–2458. https://doi.org/10.15243/jdmlm. 2020.081.2461

Saha, S., Kuehne, C., & Bauhus, J. (2014): Intra- and interspecific competition differently influence growth and stem quality of young oaks (*Quercus robur* L. and *Quercus petraea* (Mattuschka) Liebl.). Annals of Forest Science, 71(3): 381–393. https://doi.org/10.1007/s13595-013-0345-1

Salekin, S., Mason, E. G., Morgenroth, J., Bloomberg, M., & Meason, D. F. (2019): Modelling the effect of microsite influences on the growth and survival of juvenile *Eucalyptus globoidea* (Blakely) and *Eucalyptus bosistoana* (F. Muell) in New Zealand. Forests, 10(10): https://doi.org/10.3390/f10100857

Silva, L. B., Alves, M., Elias, R. B., & Silva, L. (2017): Comparison of T -Square, Point Centered Quarter, and N -Tree Sampling Methods in Pittosporum undulatum Invaded Woodlands. International Journal of Forestry Research, 2017. https://doi.org/10.1155/2017/2818132

Silva, P. S. L. e, Paiva, H. N. de, Oliveira, V. R. de, Siqueira, P. L. de O. F., Soares, E. B., Monteiro, A. L., & Tavella, L. B. (2014): Biomass of tree species as a response to planting density and interspecific competition. Revista Árvore, 38(2): 319–329. https://doi.org/10.1590/s0100-67622014000200012

Silver, W. L., Hall, S. J., & González, G. (2014): Differential effects of canopy trimming and litter deposition on litterfall and nutrient dynamics in a wet subtropical forest. Forest Ecology and Management, 332: 47–55. https://doi.org/10.1016/j.foreco.2014.05.018

Skálová, H., Jarošík, V., Dvořáčková, Š., & Pyšek, P. (2013): Effect of Intra- and Interspecific Competition on the Performance of Native and Invasive Species of Impatiens under Varying Levels of Shade and Moisture. PLoS ONE, 8(5): 1–9. https://doi.org/10.1371/journal.pone.0062842

Tetemke, B. A., Birhane, E., Rannestad, M. M., & Eid, T. (2019): Allometric models for predicting aboveground biomass of trees in the dry afromontane forests of Northern Ethiopia. Forests, 10(12): 1–15. https://doi.org/10.3390/F10121114

Truax, B., Fortier, J., Gagnon, D., & Lambert, F. (2018): Planting density and site effects on stem dimensions, stand productivity, biomass partitioning, carbon stocks and soil nutrient supply in hybrid poplar plantations. Forests, 9(6): 1–21. https://doi.org/10.3390/f9060293

Wang, H., Wan, P., Wang, Q., Liu, L., Zhang, G., & Hui, G. (2017): Prevalence of inter-tree competition and its role in shaping the community structure of a natural mongolian scots pine (*Pinus sylvestris* var. mongolica) forest. Forests, 8(3): https://doi.org/10.3390/f8030084

Washitani, I., & Tang, Y. (1991): Microsite variation in light availability and seedling growth of *Quercus serrata* in a temperate pine forest. Ecological Research, 6(3): 305–316. https://doi.org/10.1007/BF02347130

Yan, Y., Fang, S., Tian, Y., Deng, S., Tang, L., & Chuong, D. N. (2015): Influence of Tree Spacing on Soil Nitrogen Mineralization and Availability in Hybrid Poplar Plantations. 636–649. https://doi.org/10.3390/f6030636

Yang, X. zhou, Zhang, W. hui, & He, Q. yue. (2019): Effects of intraspecific competition on growth, architecture and biomass allocation of *Quercus Liaotungensis*. Journal of Plant Interactions, 14(1): 284–294. https://doi.org/10.1080/17429145.2019.1629656

Zhang, Z., Papaik, M. J., Wang, X., Hao, Z., Ye, J., Lin, F., & Yuan, Z. (2016): The effect of tree size, neighborhood competition and environment on tree growth in an old-growth temperate forest. Journal of Plant Ecology, 10(6): 970–980. https://doi.org/10.1093/jpe/rtw126

Zhou, W., Cheng, X., Wu, R., Han, H., Kang, F., Zhu, J., & Tian, P. (2018): Effect of intraspecific competition on biomass partitioning of *Larix principis-rupprechtii*. Journal of Plant Interactions, 13(1): 1–8. https://doi.org/10.1080/17429145.2017.1406999

Jalić, N., Ostojić, A., Vaško, Ž. (2022): The AHP quantification of student population attitudes in wine purchasing. Agriculture and Forestry, 68 (1): 207-217. doi:10.17707/AgricultForest.68.1.13

DOI: 10.17707/AgricultForest.68.1.13

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THE AHP QUANTIFICATION OF STUDENT POPULATION ATTITUDES IN WINE PURCHASING

SUMMARY

This paper aims to apply the methodology of the Analytical Hierarchy Process (AHP) to prioritize factors and their weighting influence on the attitudes of respondents in wine purchasing decision making. The research was conducted online using the Google forms platform. Primary data were collected from 150 students at the University of Banja Luka during a COVID-19 lock-down in the Republic of Srpska and Bosnia and Herzegovina in April 2020. Based on the collected responses in Microsoft Excel, the significant rates of the measured purchase factor were calculated. The obtained rates were used as an input variable for the Expert Choice program in which the process of determining the weight influence of attitudes on buying wine was applied. The reasoning process in this paper is based on the fuzzy method in the MATLAB R2016a program which gives a precise answer to the question of how important a given factor is when buying wine. Based on the fuzzy output, it can be concluded that wine quality factors influence the purchase decision by more than 90%. Market factors have a weighting influence on the purchase decision of less than 10%. In terms of wine quality, the most important factor is the taste of the wine, and in terms of market factors, the price.

Keywords: wine, student population, attitudes, AHP method, Fuzzy logic

INTRODUCTION

Wine production in B&H has a long tradition. As Sudarić et al. (2020) state for the example of Croatia, also in B&H viticulture and winemaking has a long tradition, a high level of production knowledge and producers experience which, in addition to favorable natural conditions and a developed market of demand, give stimulating conditions for sustainable production development. On average, about 3.500 ha are planted under vines annually, about 25.000.000 kg of grapes are harvested, from which 16.000.000 liters of wine are obtained (Ivanković et al., 2018). According to the FAO (2021), slightly less than 6 litres of wine is consumed per capita. Compared to consumption in the more developed countries

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of the world, this quantity is low and this research analyzes and identifies important factors in the consumption and purchasing of wine. Consumer behavior arises as a result of the interaction of external and internal factors, ie. various factors that act on them from the environment in which they live (Golijan, 2016). The level of understanding consumers' motives when buying wine, greatly facilitates producers and importers in the on-time and adequate organization of all activities (Vlahović et al., 2012). Nacka et al. (2016) confirmed, based on the North Macedonian National Strategy for

Viticulture and Wine production, that the wine market in the country has two consumer groups: middle-aged who have lower purchasing power and consume larger quantities of cheaper wine and younger to middle-aged with higher purchasing power who prefer smaller quantities of high-quality wine. Čavor (2015) research about consumer behavior in Montenegro research emphasized that the age of consumer play a significant role in wine purchasing - younger consumers take into consideration more attributes than older. Research in the consumer behavior in terms of attitudes and requirements in the consumption and purchasing of wine in Bosnia and Herzegovina and the city of Banja Luka has not been sufficiently researched, especially in the field of student population and Y generation (Fountain and Lamb, 2011). Because of that, the research was conducted among students of Banja Luka University. Youth people certainly do not represent the segment that has the highest consumption of wine, but it is important to explore the attitudes, opinions and preferences at the end of their adolescence (Kristić, 2012).

The decision-making process is very complex and often contains a large number of interconnected and interdependent factors whose influences are not simply precisely recognized and linked into a single decision (Srđević, 2005). The Fuzzy AHP method was applied, using the Expert Choice and MatLab R2016a programs, to determine which are the most important factors that initiate the purchase and consumption of wine by the student population at the University of Banja Luka. AHP is based on the concept of balance and is used to obtain the overall relative importance of a set of criteria/alternatives. It is applied to the analyzed decision problem involving multiple criteria at multiple hierarchical levels by assigning relative weights to the criteria and then normalizing weights using the Expert Choice program (Hadelan, 2010). The subject of this paper is the research of the application of Fuzzy AHP methods in determining the importance of quality factors and market factors based on which respondents decide to buy wine. The term wine in this paper means wine in general, red, white, imported, or domestic, so wine in the broadest sense. Recently, certain multi-criteria methods based on fuzzy logic have been used to cover a complex of problems related to group decision-making, human subjectivity, expert knowledge, and the tendency to use verbal instead of numerical grades (Srđević, 2003). Thanks to fuzzy sets and fuzzy logic, it is possible to model values that not only have to belong or do not belong but can have a certain degree of belonging to a certain set, language variable or attribute (Bašić, 2017). The values of the factors that influence the attitudes of the respondents about wines are presented by the fuzzy way of reasoning, because, as Bašić (2017) states, they describe in a more precise way the affiliation to a certain fuzzy number than the classical Boolean way of inference. Quality as the first factor in purchasing according to Zeithaml (1988) represents the overall result of experience and various influences that affect the customer, who on this basis assesses the competitiveness of product/brand quality. Jovanović et al. (2017) stated that of all the factors analyzed in relation to consumers' behavior and preference, the dominant factors identified are demographic factors-age, region, family size and place of living, social factors-education and income, and behavioral factors-price importance, place of purchase and product characteristics. The study by Radman et al. (2004) included as the most important factors of wine the name of the producer or brand, the shape of the bottle or label, the method of production, price, age, and the importance of internal (intrinsic) characteristics: color, taste and aroma. In this paper "wine quality" means color, smell, taste, alcohol content and year of harvest. The second set of factors that influence the purchase of wine and consumer attitudes when buying this product is called "market characteristics" and includes price, availability, familiarity and packaging.

MATERIAL AND METHODS

The research was conducted through the collection of primary data by a structured survey. Considering the way of data collection for this research, the method belongs to the so-called field research. The questionnaire was created by the authors and the survey was conducted on a sample of 150 students from the University of Banja Luka. Out of the total number of respondents, 5 respondents gave a negative answer to the question of whether they drink wine and therefore were not subject to further processing. The answers were collected in April 2020, online using Google forms. This method was the only one possible because of restrictions on movement caused by the COVID-19 virus.

The evaluation of the relevant weights of the obtained answers was performed based on Saaty's nine-point scale. The mentioned scale is insensitive to small changes in the expression of preferences by decision-makers, which indirectly enables good compensation of uncertainty that is often present in the process of assessing importance in pairs (Milovanović and Stojanović, 2016). Primary data collected by the structured questionnaire were sorted originally in Microsoft Office Excel. Also, in the same program, the frequencies of responses were weighted and the rates were obtained, which became inputs for the next phase of data processing using the AHP method, applied to prioritize the factors of buying wine. The weights of the response frequencies were performed to evaluate the responses as precisely as possible. Table 1 shows that the strongest weight of 0.5 was assigned to answers 1 and 9 that are furthest from the middle of the scale, a weight of 0.4 was assigned to answers 2 and 8; 0.3 answers 3 and 7; 0.2 answers 4 and 6; and answer 5 denoting the equal importance of the terms was assigned a weight of 0.1. The sum of all weighted quantities is marked as Σ_A^I.

L denotes the rate of weighted responses on the left which implies greater importance of the term on the left, answers 1, 2, 3, 4 and half of the answer 5. R denotes the rate of weighted responses on the right which implies greater importance of the term on the right, answers 6, 7, 8, 9 and a half answers 5 (Table 1).

The example of one question:

COLOUR *1 2 3 4* 5 *6 7 8 9* SMELL

L R

Table 1. Modified (weighted) Saaty scale

A) **a***0.5	An extremely important factor on the left (1-EIL)	F) **i***0.5	An extremely important factor on the right (9-EIR)
B) **b***0.4	A much more important factor on the left (2-MMIL)	G) **h***0.4	A much more important factor on the right (8-MMIR)
C) **c***0.3	A more important factor on the left (3-MIL)	H) **g***0.3	A more important factor on the right (7-MIR)
D) **d***0.2	A little more important factor on the left (4-LMIL)	I) **f***0.2	A little more important factor on the right (6-UVR)
E) **e***0.1	Neutral (5-N)	E) **e***0.1	Neutral (5-N)
LEFT SIDE OD SCALE (L)		**RIGHT SIDE OD SCALE (R)**	
1 2 3 4 5 6 7 8 9			

The frequencies of answers 1, 2, 3, 4, 5, 6, 7, 8, 9 to the question from the questionnaire represent the values (a, b, c, d, e, f, g, h, i). Values 0.5; 0.4; 0.3; 0.2; 0.1 are weights determined by the authors based on an assessment of the importance of individual responses. For example, the response frequency from surveys (a = 3; b = 2; c = 6) follows A = 3 * 0.5; B = 2 * 0.4; C = 6 * 0.3 and so on. The coefficients (Left side and Right side values) required to enter the value of each individual survey question in the Expert Choice program were obtained as follows:

$$\boldsymbol{Ls} = \frac{A + B + C + D + E/2}{\Sigma_A^I}$$

$$\boldsymbol{Rs} = \frac{F + G + H + I + E/2}{\Sigma_A^I}$$

"A, B, C, D, E, F, G, H, I" represents the Fuzzy number (FN) determined by multiplying the calculated weighted response rate on the right by the number 9:

FN = Rs*9

The Fuzzy Logic Toolbox of the MATLAB platform (R2016a) was used for the conclusion. The Fuzzy number was created on the Satty scale from the survey.

RESULTS AND DISCUSSION

The two main groups of criteria in this research are wine quality and market characteristics. As sub-criteria of quality of wine are singled out the color, smell, taste, alcohol content and year of harvest. The sub-criteria of market characteristics of the wine are price, availability, popularity and packaging. The criteria are compared according to the degree of their influence. In AHP, pairings are based on a standardized nine-level comparison scale (Saaty, 1990). Each question was set up in such a way that the respondents chose one of the answers on a nine-point scale (1 to 9).

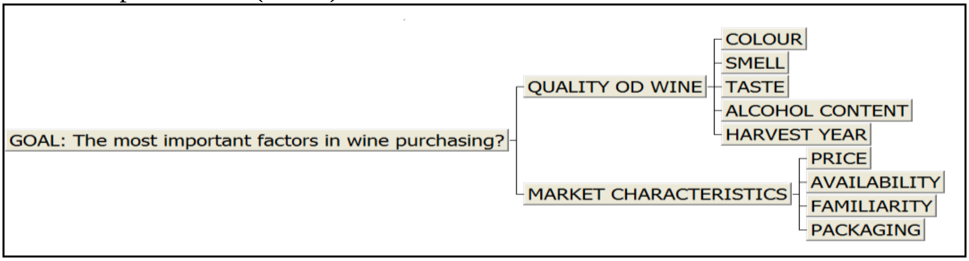

Figure 2. Hierarchy of goals, criteria and sub-criteria (Expert Choice program)

Figure 2 shows a scheme of criteria and sub-criteria that were used to examine or determine the significance of making a decision when buying wine. It is noticeable that the quality of wine and market characteristics are the two main factors, and to the right they are divided into sub-criteria. Five criteria have been determined for wine quality and four criteria for market characteristics. Presentation of the respondent's answers to the asked questions follows.

Quality as a factor when buying wine

Expert choice offers several ways to display results. The option "Synthese" is selected from the menu, (Figure 3). The sum of values corresponding to the name of the sub-criteria is 1. The highest values imply a higher influence on the quality factor. To confirm the validity of the model, the calculated inconsistency factor must be less than 0.1, which practically means that there are no logical contradictions (Ishizaka and Labib, 2009). In the case of the valuation quality factor, the inconsistency is 0.06, which means that the validity condition is met and that the comparison is valid.

Figure 3. Calculated values of the Quality sub-criteria

As Pinto et al. (2016) stated, even though the wines are very close in quality, it is possible to obtain a more precise ranking, despite the subjectivity and complexity. Based on the obtained answers of the respondents and the output of the AHP process (Figure 3), it is realized that the least important wine quality factor is its color. It affects less than 2% on wine quality. This is followed by factors of alcohol content whose significance is 5.7% and the year of harvest with a significance of 6.7%. The smell of wine is important somewhere around 10 percent for the student population. The most important factor that convincingly dominates in the attitudes of wine quality is taste, with an impact of 75.8%.

Market characteristics as a factor when buying wine

Figure 4 shows the sub-criteria of wine market characteristics and their influence on the criteria of "market characteristics". Price, availability, familiarity and packaging collectively affect 100 percent of these criteria. The factor of the inconsistency of market characteristics is 0.01 and therefore there are no logical contradictions, and the comparison is valid.

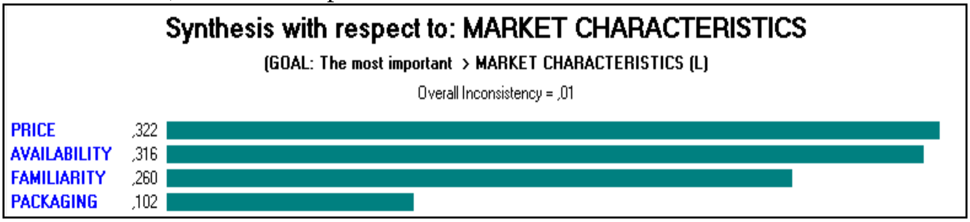

Figure 4. Calculated values of the market characteristics sub-criteria

Figure 4 shows that the least important factor in the market characteristics of wine is its packaging with a significant rate of 10%. The most important factor in this group of sub-criteria is the price of the wine with a significance of 32%. Ostojić et al. (2018) stated, comparing the changes in consumer attitudes regarding the factors that influence the decision to buy wine during the two observed periods, that price is one of the basic elements for deciding to buy wine and that 75% of respondents believe that wine prices on the domestic market are high.

Based on the weighted rates of respondents' answers, the relations between the criteria and the sub-criteria were established. This was influenced by the design of a survey questionnaire that aimed at the comparison of each of these relationships. The number of combinations (Nc) between factors, i.e. the number of questions in the questionnaire, was determined using the formula in which k represents the number of criteria (Stojanović and Regodić, 2016):

$$\mathrm{Nc} = \frac{k(k-1)}{2}$$

As an example, we wilL show the interpretation of the interconnection between responses assigned to color and smell (L = 0.16; R = 0.84) and to taste and year of harvest (L = 0.89; R = 0.11). These rates show that color affects 16% compared to smell which is 84% important. Taste is 89% important compared to 11% of the year of harvest. These relations cannot be seen directly from Figure 5 because all rates and all sub-criteria relationships (color, smell, taste, alcohol content and year of harvest) are taken into account. Sublimation of all these relations gives a percentage share of the importance of these factors. The calculated rates in the Expert Choice program serve as inputs for the Matlab Fuzzy logic toolbox or fuzzy decision-making process.

1	COLOUR	9	8	7	6	5	4	3	2	1	2	3	4	**5**	6	7	8	9	SMELL
2	COLOUR	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	**9**	TASTE
3	COLOUR	9	8	7	6	5	4	3	2	1	**2**	3	4	5	6	7	8	9	ALCOHOL CONTENT
4	COLOUR	9	8	7	6	5	4	3	2	1	2	**3**	4	5	6	7	8	9	HARVEST YEAR
5	SMELL	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	**9**	TASTE
6	SMELL	9	8	7	6	5	4	**3**	2	1	2	3	4	5	6	7	8	9	ALCOHOL CONTENT
7	SMELL	9	8	7	6	5	4	3	**2**	1	2	3	4	5	6	7	8	9	HARVEST YEAR
8	TASTE	9	8	**7**	6	5	4	3	2	1	2	3	4	5	6	7	8	9	ALCOHOL CONTENT
9	TASTE	9	**8**	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	HARVEST YEAR
10	ALCOHOL CONTENT	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	HARVEST YEAR
1	PRICE	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	AVAILABILITY
2	PRICE	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	FAMILIARITY
3	PRICE	9	8	7	6	5	4	**3**	2	1	2	3	4	5	6	7	8	9	PACKAGING
4	AVAILABILITY	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	FAMILIARITY
5	AVAILABILITY	9	8	7	6	5	4	**3**	2	1	2	3	4	5	6	7	8	9	PACKAGING
6	FAMILIARITY	9	8	7	6	5	4	3	**2**	1	2	3	4	5	6	7	8	9	PACKAGING

Figure 5. Results of crossing criteria based on the processed survey responses

Figure 5 shows the average response rates of respondents to the given comparisons. As stated in the methodology, the respondents chose between the factor on the left and the factor on the right side. The first ten comparisons refer to the quality of the wine and the criteria color, smell, taste, alcohol content and harvest year. The last six comparisons include a comparison of factors within market characteristics, price, availability, familiarity and packaging. The membership function, which can take values from the entire closed unit interval, shows how many values belong to a specific fuzzy number (Bašić, 2017). The problem of wine sensory evaluation contains many quality attributes which can not or be difficult to be depicted by crisp numbers. Linguistic terms are suitable to deal with this situation and in the real decision process, they are often transformed into triangular fuzzy numbers (Xie, 2016). Based on this and similar claims, the universal triangular fuzzy - membership functions was selected and used by which the process of defuzzification is performed, i.e. determining the degree of belonging to a certain epithet:

$$\boldsymbol{f(x;a,b,c)} = \left\{\begin{matrix} \mathbf{0} & \boldsymbol{x \le a \wedge c \le x} \\ \dfrac{\boldsymbol{x-a}}{\boldsymbol{b-a}} & \boldsymbol{a \le x \le b} \\ \dfrac{\boldsymbol{c-x}}{\boldsymbol{c-b}} & \boldsymbol{b \le x \le c} \end{matrix}\right\}$$

And by which the fuzzy functions are determined for this particular case:

$$\mu EIL = \left\{\begin{matrix} 0 & x \ge 0.125 \\ \dfrac{0.125-x}{0.125} & 0 \le x \le 0.125 \end{matrix}\right\} \quad \mu MMIL = \left\{\begin{matrix} 0 & x \le 0 \wedge 0.25 \le x \\ \dfrac{x}{0.125} & 0 \le x \le 0.125 \\ \dfrac{0.25-x}{0.125} & 0.125 \le x \le 0.25 \end{matrix}\right\}$$

$$\mu MIL = \left\{\begin{matrix} 0 & x \le 0.125 \wedge 0.375 \le x \\ \dfrac{x-0.125}{0.125} & 0.125 \le x \le 0.25 \\ \dfrac{0.375-x}{0.125} & 0.25 \le x \le 0.375 \end{matrix}\right\} \quad \mu LMIL = \left\{\begin{matrix} 0 & x \le 0.25 \wedge 0.5 \le x \\ \dfrac{x-0.25}{0.125} & 0.25 \le x \le 0.375 \\ \dfrac{0.5-x}{0.125} & 0.375 \le x \le 0.5 \end{matrix}\right\}$$

$$\mu N = \begin{Bmatrix} 0 & x \le 0.375 \wedge 0.625 \le x \\ \dfrac{x-0.375}{0.125} & 0.25 \le x \le 0.375 \\ \dfrac{0.625-x}{0.125} & 0.375 \le x \le 0.5 \end{Bmatrix} \quad \mu LMIR = \begin{Bmatrix} 0 & x \le 0.5 \wedge 0.75 \le x \\ \dfrac{x-0.25}{0.125} & 0.5 \le x \le 0.625 \\ \dfrac{0.75-x}{0.125} & 0.625 \le x \le 0.75 \end{Bmatrix}$$

$$\mu MIR = \begin{Bmatrix} 0 & x \le 0.625 \wedge 0.875 \le x \\ \dfrac{x-0.625}{0.125} & 0.625 \le x \le 0.75 \\ \dfrac{0.875-x}{0.125} & 0.75 \le x \le 0.875 \end{Bmatrix} \quad \mu MMIR = \begin{Bmatrix} 0 & x \le 0.75 \wedge 1 \le x \\ \dfrac{x-0.75}{0.125} & 0.75 \le x \le 0.875 \\ \dfrac{1-x}{0.125} & 0.875 \le x \le 1 \end{Bmatrix}$$

$$\mu \mathrm{EIR} = \begin{Bmatrix} 0 & x \le 0{,}875 \\ \dfrac{x-0{,}875}{0{,}125} & 0{,}875 \le x \le 1 \end{Bmatrix}$$

EIL means extremely important on left, MMIL-much more important on left, MIL-more important on left, LMIL-little more important left, N-neutral, LMIR-little more important right, MIR-more important right, MMIR-much more important right, EIR-extremely important on the right side.

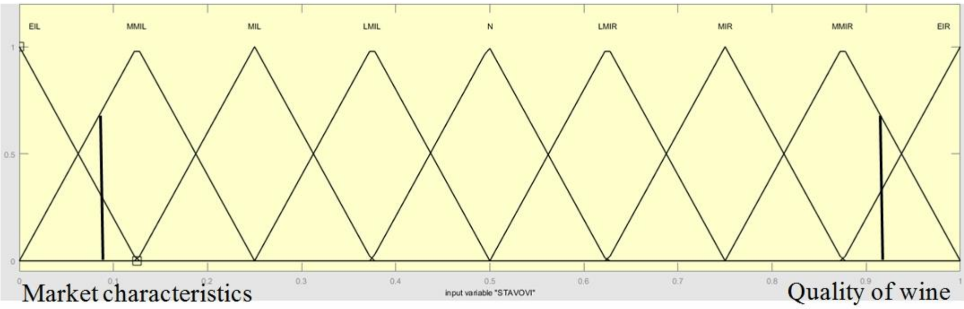

Figure 6. Determining the fuzzy value

Figure 6 shows the degree of belonging to the epithets of the variables, Quality (Q) and Market Characteristics (MC) that were compared. The values of these epithets were obtained based on the Expert Choice methodology and then used as inputs in the fuzzy process. Calculated values are 0.916 for quality and 0.084 for market characteristics. Based on the set of fuzzy functions of belonging to epithets, the degrees of belonging to each epithet are determined, and therefore the quality belongs to the interval from 0.875 to 1. For this interval, two functions of fuzzy belonging to the epithet quality are determined, the first Qmmir= $\frac{1-x}{0{,}125}$, and second Qeir=$\frac{x-0{,}875}{0{,}125}$. The calculation gives the values: Quality is 67.2% much more important (MMI) and 32.8% extremely important (EI) than the criterion with which it is compared. Belonging to certain epithets is determined in the same way for another criterion, market characteristics. With a value of 0.084, it is in the range of 0 to 0.125. Fuzzy functions are defined for this interval MCmmil=$\frac{x}{0{,}125}$ i MCeil=$\frac{0{,}125-x}{0{,}125}$.

The calculation determined that market characteristics are extremely unimportant than quality 32.8% and much more unimportant than quality 67.2%.

Consumers could be viewed from different aspects, as an individual, member of some social group or class, or as a representative of a certain nation, race, or religion, or also a person who buys a certain product to satisfy personal needs, existential safeness and social acceptance, and even to show the prestige over other members of the social community (Vlahović et al., 2012). In this case, consumers were considered as a person who buys some product for the exact reasons.

The ratio of Quality (Q) of wine and Market Characteristics (Q) was (L = 0.916; R = 0.084), which means that for the respondents the Wine Quality factor is important 91.6% in relation to the Market Characteristics, which are important only 8.4%. So, the factors color, smell, taste, alcohol content and year of harvest have a weight impact of 0.916 on buying wine, while the factors of price, availability, familiarity and packaging have a weight impact of 0.084. This attitude stems from the fact that the students population does not earn their income, so the money is worthless. Based on that, price and market factors have a smaller influence on the decision to wine purchase.

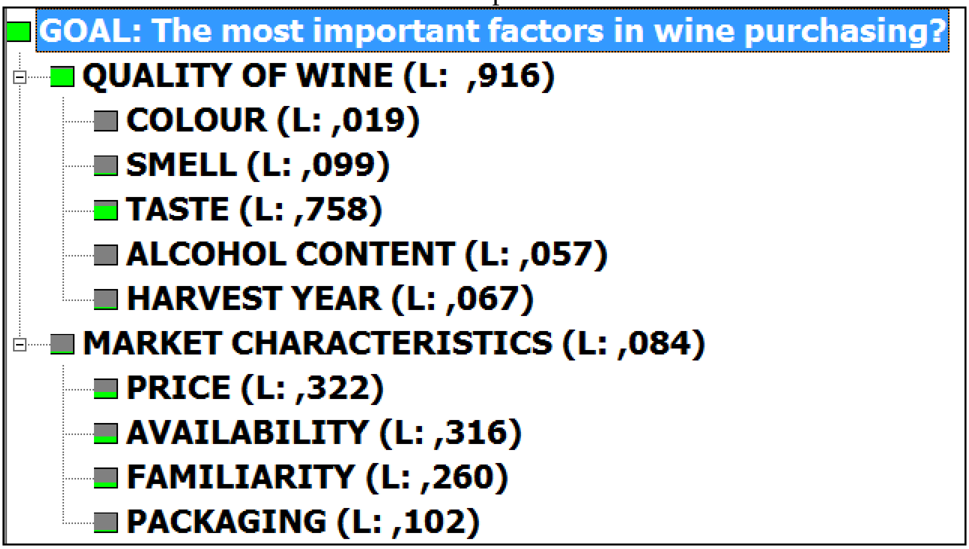

Figure 7. Calculated values of criteria and sub-criteria (Expert Choice program)

The conducted research highlights the limitations and possibilities for further research. That is, above all, a question why the price is not as important as expected in the purchase preferences of the student population. For the following research, we recommend that the compared factors be in the same plane, i.e. that there are no more levels of criteria and sub-criteria. In this way, all factors would be directly compared with each other and the influence of potential ignorance that the sub-criteria make up certain criteria would be removed.

In addition to these, the following research could expand and increase the sample of respondents as well as the method of data collection, preferring a face-to-face survey instead of an online survey.

CONCLUSIONS

The aim of the research was to show the factors that are important for student populations in Banja Luka when buying wine. Likewise, the goal was to try to merge the Analytical Hierarchical Process and the Fuzzy process. Based on the Expert Choice program, the influence of individual sub-criteria on the main criteria was determined, and then the influence of the two main criteria on the final purchase decision. By weighing the main criteria, the influence of the sub-criteria on the final purchase decision was determined. The quality of the wine for the student population is a much more important factor than the market characteristics and assigned about 91% of the influence on the purchase. As the least important factors, students evaluate colour and packaging. The following less important factor is the familiarity of wines with a small impact on wine purchasing. Price and availability equally impact on purchase, less than 3%. The alcohol content participates with 5.2%, harvest year with 6.1% on the purchase of a particular wine. The smell is 9.1% important and the absolute most dominant criteria for buying wine is a taste of wine with an impact of more than 60%.

REFERENCES

Bašić, B. (2017): Uloga fuzzy skupova u rangiranju objekata, Univerzitet u Novom Sadu, Prirodno-matematički fakultet, Master thesis.

Čavor, T. (2015): Consumer behavior on Montenegrin wine market. Master's thesis, University of Ljubljana, Faculty of Economics.

FAO (2021): Faostat data, available on: http://www.fao.org/faostat/en/#home, accessed on 28.08.2021.

Fountain, J., Lamb, C. (2011): Generation Y as young wine consumers in New Zealand: how do they differ from Generation X?, International Journal of Wine Business Research 23, 107-124.

Golijan, J. (2016): Motivi koji utiču na kupovinu organskih prehrambenih proizvoda, *Agroekonomika*, Novi sad, 45 (72), 73-80.

Hadelan, L. (2010): Višekriterijsko odlučivanje u poredbenoj analizi sustava proizvodnje kupine, Sveučilište u Zagrebu, Agronomski fakultet, doktorska disertacija

Ishizaka, A., Labib, A. (2009): Analytic hierarchy process and expert choice: benefits and limitations. OR Insight, 22 (4), 201-220.

Ivanković, M., Beljo, M., Prusina, T. (2018): Ekonomska i društvena uloga vinogradarstva i vinarstva u Bosni i Hercegovini, Proceedings of the international scientific meeting "130 godina organiziranoga vinogradarstva i vinarstva u Bosni i Hercegovini", Mostar, Bosna i Herzegovina, 374-391.

Jovanović, M., Kašćelan, Lj., Joksimović, M., Kašćelan, V. (2017): Decision tree analysis of wine consumers' preferences: evidence from an emerging market, *British Food Journal,* 119(6): 1349-1361.

Kristić, J. (2012): Stavovi studentske populacije o utjecaju promocijskog spleta na kupnju vina, Ekonomski vjesnik: Review of Contemporary Entrepreneurship, *Business and Economic Issues*, 15 (1), 98-105.

Milovanović, Ž., Stojanović, M. (2016): Izbor sorti višnje za sadnju primenom ahp metodologije, *Agroekonomika*, Novi Sad, 45 (72), 11-19.

Nacka, M., Georgiev, N., Simonovska, A. (2016): Young consumers' preferences for Macedonian wine, *Agriculture and Forestry*, 62(2): 103-110.

Pinto, F., Marques, G., Morim, A. (2016): An AHP Application to Wine Evaluation: Rating Based on the Criteria Framework of the Method Adopted by Brazilian Sommeliers Association - abs., *International Symposium on the Analytic Hierarchy Process, London, U.K., DOI:* 10.13033/isahp.y2016.021.

Radman, M., Kovačić, D., Gašparec-Skočić Lj. (2004): Percepcija i potrošnja vina među mladima u Hrvatskoj, *XXVIII. World Congress of Vine and Wine 84th General Assembly of the OIV, 4 – 9 July 2004, Vienna.*

Srđević, B. (2003): Metodi i rešenja višekriterijumske analize u poljoprivredi. Agroekonomika 32. Univerzitet u Novom Sadu, Poljoprivredni fakultet, 307-312.

Srđević, B. (2005): Diskretni modeli odlučivanja u optimizaciji korišćenja kanalske mreže u Vojvodini, *Letopis naučnih radova,* 29 (1), 19-30.

Stojanović, M., Regodić, D. (2016): Ocenjivanje sajtova za E-kupovinu u Srbiji upotrebom AHP-TOPSIS metoda, *Zbornik radova Univerziteta Sinergija*, 1 (1), 99-104

Sudarić, T., Samardžija, L., Lončarić, R. (2020): Viticulture and wine as export potential of Croatia, *Agriculture and Forestry*, 66(2), 57-66.

Vlahović, B., Potrebić, V., Jeločnik, M. (2012): Preferences of wine consumers on Serbian market, Economics of agriculture, 59 (1), 37-49.

Xie, J. (2016): A Novel Fuzzy Mathematical Method to the Sensory Evaluation of Wine, *Advance Journal of Food Science and Technology 10(5): 343-347. DOI: 10.19026/ajfst.10.2079.*

Zeithaml, V.A. (1988): Consumer Perceptions of Price, Quality, and Value: A Means-End Model and Synthesis of Evidence, *Journal of Marketing,* 52(3), 2-22.

Schwartz, G., da Silva Rocha, T., Sá de ALmeida, M. J., Silva Dionisio, L. F., Corvera, R. (2022): Seedlings quality of Bertholletia excelsa Bonpl. (Lecythidaceae) produced in forest nursery, Agriculture and Forestry, 68 (1): 219-232. doi:10.17707/AgricultForest.68.1.14

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SEEDLINGS QUALITY OF *BERTHOLLETIA EXCELSA* BONPL. (LECYTHIDACEAE) PRODUCED IN FOREST NURSERY

SUMMARY

In this study, the quality of *Bertholletia excelsa* seedlings was evaluated in relation to the transplanted seedling's size. Thirty days after transplanting the seedlings to the forest nursery, the first height measurement was performed. From the 30th day onwards, evaluations were carried out every 15 days, being nine in total. The Dickson Quality Index (DQI) was evaluated to determine the seedlings quality, where all seedlings had their root pruned. The experimental design, totaling 160 individuals, was completely randomized with four treatments and four replications, including the shoot cut and three seedling sizes with no shoot cut. Shoot cut seedlings presented a DQI of 0.66 ± 0.23, significantly higher than the large seedlings (≤ 17 cm in height) with no shoot cut ($F_{3,76}$ = 2.762, p = 0.047). Transplanting had significant effects over the development, growth, and quality of *Bertholletia excelsa* seedlings during the first 165 days of seedling production. Therefore, shoot pruning resulted in better performance of *B. excelsa* seedlings to be planted in the field.

Keywords: Brazil nut, seedling transplanting, seedling production, native tree species, Peruvian Amazon

INTRODUCTION

Bertholletia excelsa Bonpl., commonly known as Brazil nut, is a tree species of the family Lecythidaceae with great economic importance in the

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Amazonian region (Albuquerque et al. 2015; Nogueira et al. 2018). Its nuts are a non-timber forest product that brings employment and income for thousands of workers in Bolivia, Brazil, and Peru through the seed's market (Scoles et al. 2016, Costa et al. 2015). B. excelsa seeds have high nutritional value and normally attain high commercial value in domestic and international markets (Baldoni et al. 2017, Wadt et al. 2018).

Bertholletia excelsa is a climax and light-demanding species that presents good performance in growth when planted in open areas (Scoles et al. 2011, Albuquerque et al. 2015). Adult trees present large size, reaching 40-60 m in height and 1-4 m in diameter (Santos et al. 2006). In natural environments, B. excelsa tend to present local high densities, because the individuals tend to be aggregated (Mori and Prance 1990).

Seedlings production of tree species is an essential activity to build forest stands, as seedling quality influences the tree development in the field (Dionisio et al. 2019a). Seedling survival under field conditions is a crucial phase in planting, which can be determined by how the seedlings were produced in forest nurseries (Dionisio et al. 2020). The need to produce seedlings both for forest restoration and for commercial purposes requires knowledge, skills, and sensitivity from nurserymen to seek techniques to produce high quality seedlings under low costs (Dionisio et al. 2019b). In this case, seedlings must have characteristics that support the maximum survival rate and rapid initial growth after planting (Auca et al. 2018, Souza et al. 2019).

Currently, there are few recommendations and prescriptions about nursery cultivation of native tree species of the Amazon, as well as the use of alternative containers and substrates that can provide better growth and quality for B. excelsa seedlings. One of the hurdles faced during the process of seedling production of Amazonian native tree species is that many of them are slow-growing species. Furthermore, suitable containers and substrates are also a key to attain high quality seedlings produced in forest nurseries. However, a cultivation system for B. excelsa has not yet been defined, which demands the development of new technologies for that (Auca et al. 2018). Among the factors that influence the production process of tree species seedlings, there are: kind of substrate, substrate moisture, substrate porosity, seeds dormancy, temperature, shading and container volume, irrigation, seed quality, fertilization, and seedling management in the nursery (Camargo et al. 2011, Costa et al. 2015, Marques et al. 2018).

Regarding cultivation practices to optimize seedling production, the benefits of the transplanting process with pruning can be attained with more robust seedlings and better development balance in height and root system. As for the containers, acquisition cost, durability, easy handling, storage, transport, and availability in the market must be considered in seedling production of native tree species. The container's size should be chosen to provide the largest possible volume of substrate to the roots, but with low weight for an easier transport to the field. In this sense, associating nutritional power with the use of smaller containers can be a way to reduce production cost, transport, and field distribution, providing greater efficiency in planting operations (Pinho et al. 2018, Lima Filho et al. 2019).

Currently, there are almost no studies on transplanting methods and techniques to improve seedling quality of B. excelsa. Thus, the objective of this study was to test the initial growth and quality B. excelsa seedlings of different sizes in forest nursery.

MATERIAL AND METHODS

The experiment was carried out in the forest nursery of the Peruvian Amazon Research Institute (IIAP), located in the Department of Madre de Dios, Peru (12º39'04" S, 69º19'17" W) between June and September 2018. Nuts of *Bertholletia excelsa* were collected from the ground around their mother trees in forest concessions of the Peruvian Amazon and manually processed with a machete.

Figure 1. Treatments of shoot pruning (T1); small size individuals, ≤ 5 cm in height (T2); medium size individuals, ≤ 11 cm in height (T3); and large size individuals, ≤ 17 cm in height, (T4) used to assess initial growth and quality of *Bertholletia excelsa* seedligns. All seedlings had root pruned.

Table 1. Treatments used to assess initial growth and quality of *Bertholletia excelsa* seedligns during 165 days in forest nursery, Puerto Maldonado, Peru, 2018.

Treatments	**Repetitions/individuals**
Shoot cut (T1)	4/10
Small individuals (≤ 5 cm in height)/ no shoot cut (T2)	4/10
Medium individuals (≤ 11 cm in height)/ no shoot cut (T3)	4/10
Large individuals (≤ 17 cm in height)/ no shoot cut (T4)	4/10

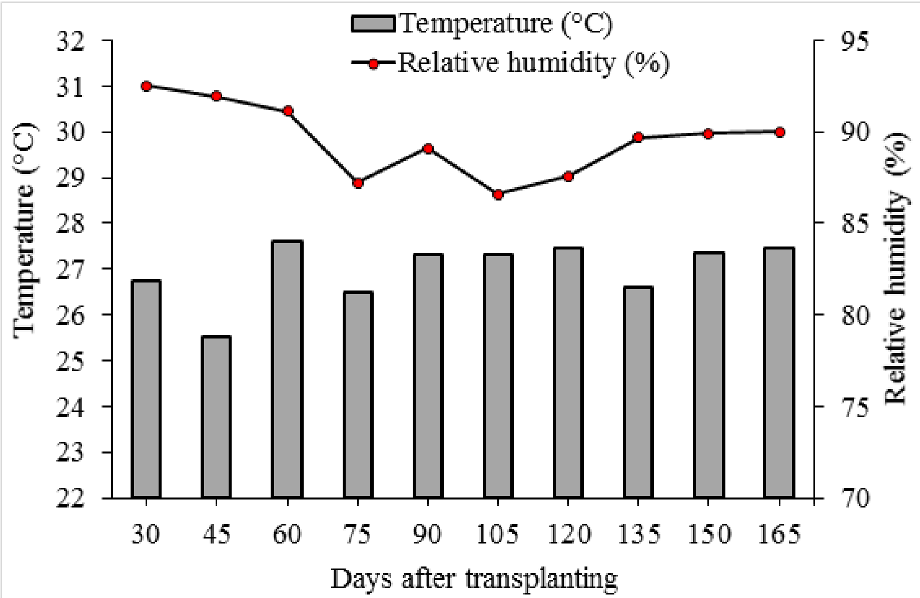

Figure 2. Average values of temperature and relative humidity during the 165 days of the experiment evaluation, Puerto Maldonado, Peru, 2018.

Seeds were immersed in water for 30 days to remove woody integument, and then a lathe was used to remove the bark. Almonds were treated with fungicide Vitavax 300 for 2 h and dried under shade during 1 h. A masonry seedbed measuring 10 m x 1 m x 0.5 m (length, width, and height) was used for sowing. Washed sand was used as substrate for seed germination in the seedbed. The sand was disinfected with boiled water (100 ºC) in 200-liter drums, and 24 h after disinfestation, almonds were sowed. The seedbed was covered with 60% shading mesh at 1 m in height and irrigation was carried out twice a day (morning and afternoon).

A total of 160 *B. excelsa* seedlings had their roots pruned and were transplanted. Besides root pruning of all individuals, the seedling were divided in four treatments as described in Figure 1 and Table 1. Each treatment had four repetitions of 10 seedlings per treatment (Table 1). To evaluate the initial growth, *B. excelsa* seedlings were selected 30 days after germination and transplanted into 115-cm^3 tubes with substrate composed of sand, sawdust, and carbonized sawdust in proportions (1:1:1 v/v). Plants were previously standardized by size and treatments were subsequently established (Table 1).

Thirty days after transplanting seedlings to the forest nursery, the first length measurement was performed. From the 30th day onwards, evaluations were carried out every 15 days, totaling nine evaluations (45, 60, 75, 90, 105, 120, 135, 150, and 165 days). A Data Logger was used for daily measurement of temperature and humidity during the experiment evaluation. Eight daily measurements were performed (1-h intervals between each measurement), where the averages are shown for every two weeks in Figure 2.

Initial seedling growth and seedling quality indexes

Seedlings initial growth was evaluated through the following variables: a) shoot length (SL), b) root length (RL), c) stem collar diameter, d) root collar diameter, e) shoot dry mass (SDM), f) root dry mass (RDM), g) total dry mass (TDM), h) shoot length/root length (SL/RL) ratio, i) shoot dry mass/root dry mass (SDM/RDM) ratio, j) robustness index (RI), k) lignification index (LI), and m) the Dickson Quality Index (DQI). The higher is DQI, RI, and LI, the greater is seedling quality. To measure the stem collar diameter, a digital caliper (accuracy = 0.01 mm) was used and to measure the shoot length and root length, a ruler graduated in millimeters was used.

To assess the seedlings dry mass, the individuals were split into shoots and roots by cutting at the point of the stem collar diameter. Both parts were placed in Kraft paper bags, identified and dried in oven at 70 °C for 72 hours until reaching constant mass. Immediately after the oven, they were weighed on an analytical balance (accuracy = 0.001 g) to obtain the shoot and root dry mass.

The shoot length/root length (SL/RL) ratio predicts the seedling success in a balance between the plant's shoot and the root system. It is determined by the following equation:

$$SL/RL = \frac{Shoot\ length\ (cm)}{Root\ length\ (cm)}$$

The ratio between shoot dry mass/root dry mass (SDM/RDM) indicates the seedling development in nurseries. It is given by the equation:

$$SDM/RDM = \frac{Shoot\ dry\ mass\ (g)}{Root\ dry\ mass\ (g)}$$

The robustness index (RI) was calculated as the ratio between the shoot length and root collar diameter, as follows:

$$RI = \frac{Shoot\ length\ (cm)}{Root\ collar\ diameter\ (mm)}$$

The lignification index (LI) relates the total dry mass with the total wet mass, giving the percentage of lignification. It is determined by the equation:

$$LI = \left(\frac{Total\ dry\ mass\ (g)}{Total\ wet\ mass\ (g)}\right)\ x\ 100$$

The Dickson Quality Index (DQI) brings together several morphological characteristics into a single value given by the following equation:

$$DQI = \frac{Total\ dry\ mass\ (g)}{\frac{Shoot\ length\ (cm)}{Stem\ collar\ diameter\ (mm)}\ \frac{Shoot\ dry\ mass\ (g)}{Root\ dry\ mass\ (g)}}$$

Data analysis

The experimental design applied was completely randomized with four treatments and four replicates of 10 seedlings each (Table 1). To verify the assumptions of the analysis of variance (ANOVA), the data were first checked for: a) normality with the Shapiro-Wilk test ($p > 0.05$), b) homoscedasticity by the Bartlett test ($p > 0.05$), and the independence between experimental units.

Cumulative and relative growth rate during 165 days were submitted to ANOVA and subsequent regression analysis, adjusting the equations to the data obtained as a function of time after transplantation in each treatment. The model for each variable was selected considering the significance of the coefficients of the variables and the highest coefficient of determination (R^2). Tukey's post-hoc test was used to test the regression coefficients ($p < 0.05$). Once these assumptions were met, the data were submitted to ANOVA using the software R, version 3.5.2 and, in case of significant differences, averages were compared by Tukey's post-hoc test ($p < 0.05$).

RESULTS

A higher initial cumulative growth in height of *Bertholletia excelsa* seedlings was observed in the treatment of medium (27.6 cm) and large size individuals (27.7 cm) (Figure 3A). Regarding the relative growth rate (Figure 3B), the shoot cut treatment presented higher cumulative growth, which decreased over time. The treatments small, medium, and large size individuals obtained lower relative growth rate, respectively (Figure 3).

Medium (31.3 ± 4.2 cm) and large (30.7 ± 2.8 cm) individuals had the highest means for shoot length, differing significantly from the other treatments ($F_{3,76} = 19.55$, $p = 0.001$), however, they did not differ significantly ($p = 0.923$) each other (Figure 4A). Regarding root length, the treatment shoot cut had the highest mean (14.1 ± 0.7 cm; $F_{3,76} = 5.621$, $p = 0.002$). Small, medium, and large individuals showed no significant difference among them in root length (Figure 4B). Shoot cut individuals presented the largest stem collar diameter (6.0 ± 1.0; Figure 4C) and in root collar diameter (Figure 4D).

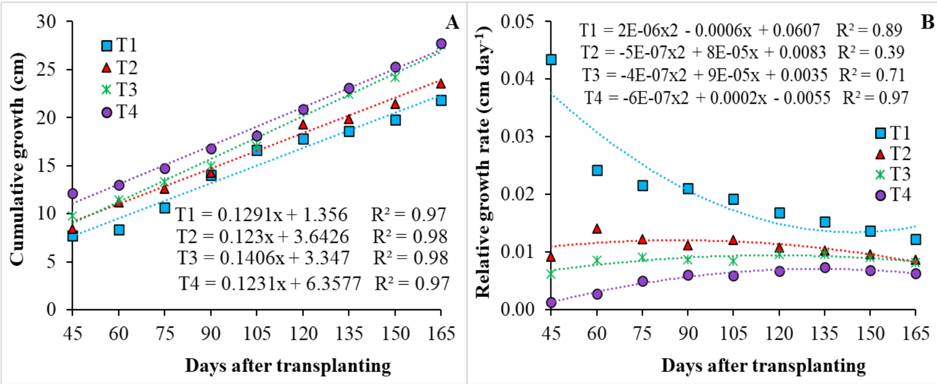

Figure 3. Cumulative growth (A) and relative growth rate in height (B) of *Bertholletia excelsa* seedlings under different sizes as a function of time after root cut and transplanting. Shoot pruning (T1); small size individuals, ≤ 5 cm in height (T2); medium size individuals, ≤ 11 cm in height (T3); and large size individuals, ≤ 17 cm in height, (T4).

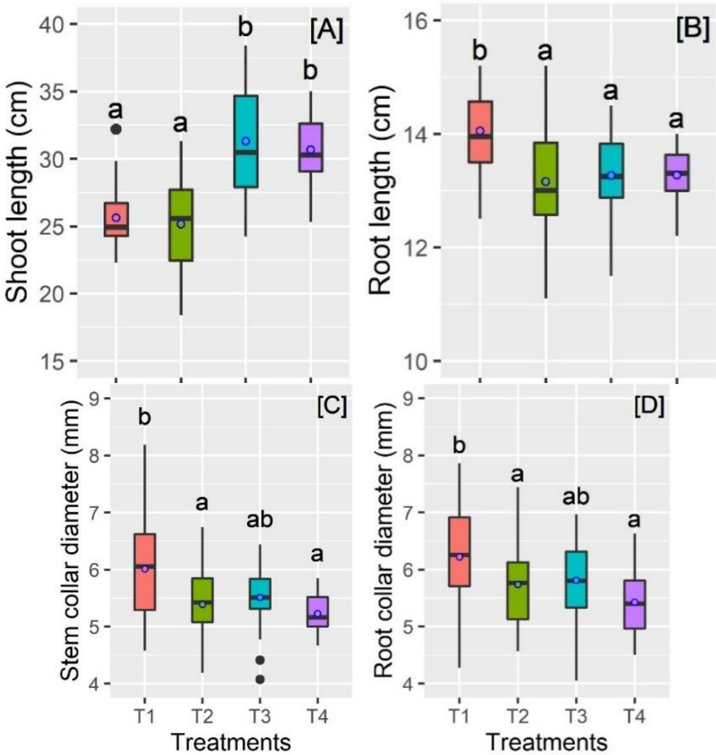

Figure 4. Shoot length (A), root length (B), stem collar diameter (C), and root collar diameter (D) of *Bertholletia excelsa* seedlings under different sizes after root cut and transplanting. Shoot pruning (T1); small size individuals, ≤ 5 cm in height (T2); medium size individuals, ≤ 11 cm in height (T3); and large size individuals, ≤ 17 cm in height, (T4). The thicker horizontal line represents average, the box the interquartile range and the dashed lines the extreme values. Letters indicate statistical differences (p = 0.05) in ANOVA with Tukey's post-hoc test.

The variables shoot dry mass, root dry mass, and total dry mass did not present significant differences among treatments (Figure 5). The shoot length/root length (SL/RL) ratio presented highest averages in medium (2.4 ± 0.3) and large (2.3 ± 0.2) individuals, which differed significantly from treatments shoot cut and small individuals (p = 0.001) (Figure 5D). The shoot dry mass/root dry mass (SDM/RDM) ratio showed no significant difference among treatments (Figure 5E).

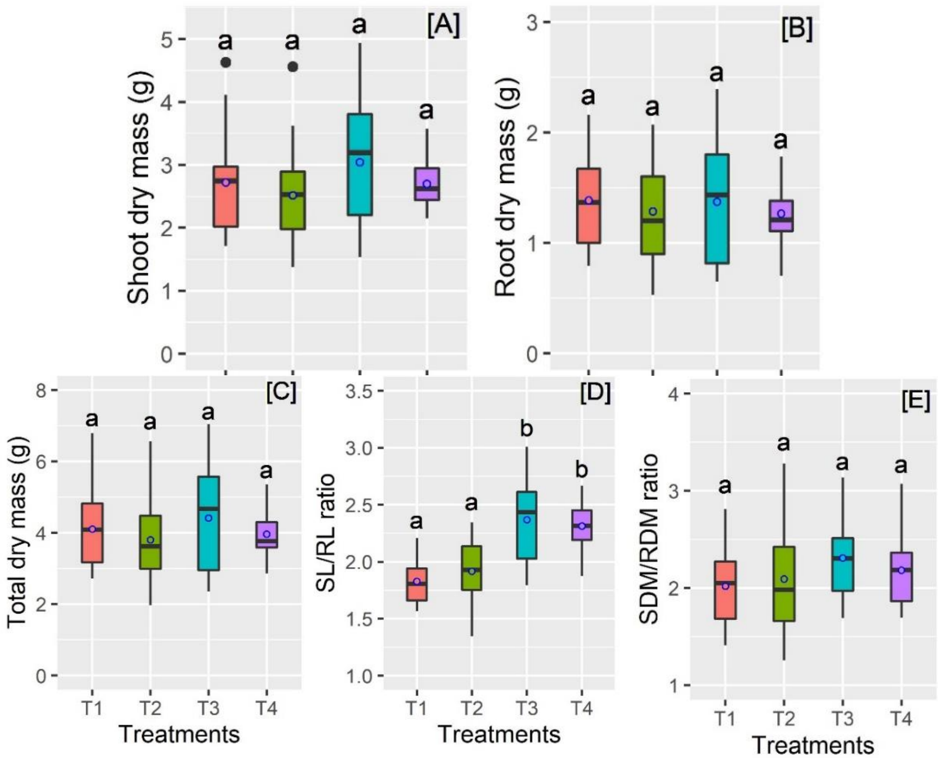

Figure 5. Shoot dry mass (A), root dry mass (B), total dry mass (C), shoot length/root length (SL/RL) ratio (D), and shoot dry mass/root dry mass (SDM/RDM) ratio (E) of *Bertholletia excelsa* seedlings under different sizes after root cut and transplanting. Shoot pruning (T1); small size individuals, ≤ 5 cm in height (T2); medium size individuals, ≤ 11 cm in height (T3); and large size individuals, ≤ 17 cm in height, (T4). The thicker horizontal line represents average, the box the interquartile range and the dashed lines the extreme values. Letters indicate statistical differences (p = 0.05) in ANOVA with Tukey's post-hoc test.

Seedlings quality indexes

Medium and large individuals had highest averages (5.4 ± 0.8 and 5.7 ± 0.7, respectively) of the robustness index, differing significantly from treatments shoot cut (4.2 ± 0.9) and small individuals (4.5 ± 0.6) ($F_{3,76}$ = 17.36, p = 0.001) (Figure 6A). The treatment shoot cut presented highest mean of lignification index (0.43 ± 0.03, $F_{3,76}$ = 11.18, p = 0.001), differing significantly from the other treatments (Figure 6B). In terms of the Dickson Quality Index (DQI), there was a difference between shoot cut (0.66 ± 0.23) and large (0.50 ± 0.09) individuals ($F_{3,76}$ = 2.762, p = 0.047) (Figure 6C).

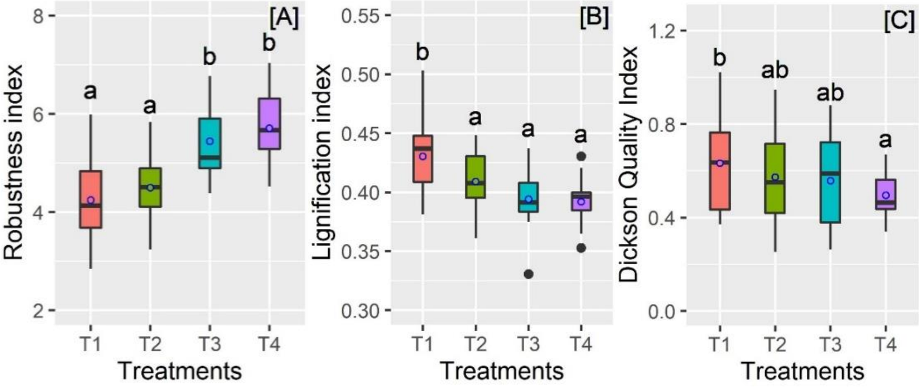

Figure 6. Robustness index (A), lignification index (B) and the Dickson Quality Index (C) of *Bertholletia excelsa* seedlings under different sizes after root cut and transplanting. Shoot pruning (T1); small size individuals, ≤ 5 cm in height (T2); medium size individuals, ≤ 11 cm in height (T3); and large size individuals, ≤ 17 cm in height, (T4). The thicker horizontal line represents average, the box the interquartile range and the dashed lines the extreme values. Letters indicate statistical differences (p = 0.05) in ANOVA with Tukey's post-hoc test.

Table 2. Morphological and qualitative variables of *Bertholletia excelsa* seedlings after transplantation under different sizes and root cut. Shoot pruning (T1); small size individuals, ≤ 5 cm in height (T2); medium size individuals, ≤ 11 cm in height (T3); and large size individuals, ≤ 17 cm in height, (T4).

Variable	**Averages**				**Quality and Interval***		
	T1	T2	T3	T4	Large	Medium	Small
Height (cm)	25.6	25.2	31.3	30.7	15-25	10-14.9	< 10.0
Stem colar diameter (mm)	6.0	5.4	5.5	5.2	≥ 4.0	2.5-3.9	< 2.5
SL/RL ratio	1.8	1.9	2.4	2.3	≤ 2.0	2.1-2.5	> 2.5
SDM/RDM ratio	2.0	2.1	2.4	2.2	1.5-2.0	2.1-2.5	> 2.5
Lignification index	0.43	0.41	0.39	0.39	nd.	nd.	nd.
Robustness index	4.2	4.5	5.4	5.7	≤ 6.0	6.1-8.0	> 8.0
Dickson Quality Index	0.66	0.57	0.56	0.50	≥ 0.5	0.49-0.20	< 0.20

*For conifer species (Reyes *et al.* 2014)
Nd. = Not determined

Optimal intervals were determined to qualify the morphological variables of *B. excelsa* seedlings in forest nursery (Table 2). Regarding the seedling quality, all tested treatments resulted in values equal to or higher than those proposed in the literature. However, the treatment shoot cut attained the highest DQI.

DISCUSSION

Shoot cut (pruning) of the *Bertholletia excelsa* seedlings at the time of the transplanting did not influence the seedlings development and quality, even though the significant effects over some variables such as stem collar diameter and root length (Figure 4 and Table 2). Shoot pruning consists of eliminating part of the seedling's terminal sprout, changing the seedling growth rate. Shoot pruning can favor seedling development, however, it depends on the tolerance level of each species.

Shoot pruning at the time of transplanting the seedlings to the definitive containers can obtain benefits such as: more robust seedlings and an adequate balance of development in shoot and root system. Precocity and uniformity are important characteristics in the seedling production, since the longer the seedlings remain in the forest nursery, higher is the production cost (Auca *et al.* 2018). Production cost is an important variable in seedling production. The need to reduce costs led managers to seek technologies to improve the way how seedlings are produced in nurseries (Ribeiro *et al.* 2018). Environmental factors and silvicultural techniques used are crucial to reduce costs and time to establish a tree plantation (Lima Filho *et al.* 2019).

The SDM/RDM ratio indicates biomass distribution between the shoot and root system (Siqueira *et al.* 2018). Thus, it is important that the container favors the seedling shoot and root growth, so that there is not a big difference between these two parts of the plant. Low SDM/RDM ratio indicates that seedlings have equal distribution of dry matter between shoot and root (Marana *et al.* 2015). The balanced distribution between shoot dry mass and root dry mass allows for adequate plant development, reducing the risk of plant toppling in the field.

Substrate temperature depends on the tube’s volume, varying according to time and space, with an important role in physical processes and energy exchanges with the surrounding environment, which interfere in the shoot and root growth and in the absorption of water and nutrients (Cavalcanti *et al.* 2019). Furthermore, the substrate temperature determines evaporation and aeration, as well as the speed of the chemical reactions involved seedling development (Diniz *et al.* 2014, Nascimento *et al.* 2016). For the production of high quality seedlings it is necessary to use suitable containers, where volume is a crucial variable. Therefore, the container size should be chosen to provide the largest possible volume of substrate to the roots, but with less weight to make the transport to the field easier (Pinho *et al.* 2018).

Seedlings quality indexes

Plant quality is a concept mainly based on morphological and physiological characteristics, which determine its survival and initial growth, depending on local conditions where the individual will be planted. Significant differences were observed for the lignification, robustness, and the Dickson Quality Index (DQI) as a function of seedling size. The robustness index is the relationship between shoot length and root collar diameter, the lower its value, the more robust is the plant. Plants produced in 345-cm³ tubes, for example, have lower robustness index, thus with better quality. This index is an indicator of plant resistance to wind desiccation, survival, and potential growth in dry sites (Reyes *et al.* 2014). The relationship between shoot length and root collar diameter is a non-destructive evaluation method to assess the balance between seedling growth and seedling quality.

The lignification index is related to the lignification of aerial and root tissues of seedlings belonging to woody species under stress. The physical stimulus applied in the form of stem flexion triggers morphometric responses in plants normally associated with a reduction in height, increase in stem diameter and in the dry matter mass of the root tissue (Dranski *et al.* 2015). The response to mechanical stimulus is desirable during the adaptation phase of seedlings to the field. The lignification of seedling tissues has been related to field performance and survival (Dranski *et al.* 2015).

The DQI, as well as the lignification index, are indicators of seedling quality, where the DQI considers vigor and balance of biomass distribution in the seedling (Lima Filho *et al.* 2019). Several authors consider the DQI as the main indicator of seedling quality, as its interpretation considers the robustness and balance of biomass distribution in the seedling, combining growth variables and biometric relationships (Binotto *et al.* 2010, Siqueira *et al.* 2018). Binotto *et al.* (2010) observed that dry mass, followed by stem diameter, are the most strongly variables correlated with the DQI.

Seedling stage is possibly the most vulnerable stage during the plant's life cycle. Under natural conditions, seedling establishment and subsequent reproductive success can be hampered for multiple reasons: premature emergence, germination at improper depths, and emergence in environments with high competitive pressure to survive or leave offspring. Thus, seeds must have effective mechanisms to detect changes in environmental conditions, in order to be able to establish the development of new stages of life (Batlla and Benech-Arnold 2014).

CONCLUSIONS

Shoot cut (pruning) did not present negative effects over the development, growth, and quality of seedlings of the Amazonian tree species Bertholletia excelsa during the first 165 days of seedling production in forest nursery. Seedlings that had the shoot cut presented higher Dickson Quality Index than seedling with no shoot cut, regardless of the initial size of the individuals transplanted. Therefore, shoot cut implies in better performance of B. excelsa seedlings to be planted in the field.

REFERENCES

Albuquerque, T. C. S., Evangelista, T. C., & Albuquerque Neto, A. A. R., (2015). Níveis de sombreamento no crescimento de mudas de castanheira do Brasil. *Revista Agro@mbiente On-line*, 9(4), 440-445. DOI: http://dx.doi.org/10.18227/1982-8470ragro.v9i4.3025

Auca, E. C., Dionisio, L. F. S., Lozano, R. M. B., & Schwartz, G., (2018). Propagation of Brazil nut (Humb. y Bonpl) seedlings using seeds in mini-greenhouses. *Revista Agro@mbiente On-line*, 12(4), 300-313. DOI: https://doi.org/10.18227/1982-8470ragro.v12i4.5222

Baldoni, A. B., Wadt, L. H. O., Campos, T., Silva, V. S., Azevedo, V. C. R., Mata, L. R., Botin, A. A., Mendes, N. O., Tardin, F. D., Tonini, H., Hoogerheide, E. S. S., & Sebbenn, A. M., (2017). Contemporary pollen and seed dispersal in natural populations of *Bertholletia excelsa* (Bonpl.). *Genetics and Molecular Research*, 16(3), gmr16039756. DOI: https://doi.org/10.4238/gmr16039756

Batlla, D. & Benech- Arnold, R. L., (2014). Weed seed germination and the light environment: Implications for weed management. *Weed Biology and Management*, 14(2), 77-87. DOI: https://doi.org/10.1111/wbm.12039

Binotto, A. F., Lúcio, A. Dal C., & Lopes, S. J., (2010). Correlations between growth variables and the Dickson quality index in forest seedlings. *Cerne*, 16(4), 457-464. DOI: https://doi.org/10.1590/S0104-77602010000400005

Camargo, R., Pires, S. C., Maldonado, A. C., Carvalho, H. P., & Costa, T. R., (2011). Avaliação de substratos para a produção de mudas de pinhão-manso em sacolas plásticas. *Revista Trópica – Ciências Agrárias e Biológicas*, 5(1), 31-38. DOI: http://dx.doi.org/10.0000/rtcab.v5i1.292

Cavalcanti, S. D. L., Gomes, N. F., Pandorfi, H., Almeida, G. L. P., & Montenegro, A. A. A., (2019). Variação espaço-temporal da temperatura do substrato em bandejas de produção de mudas. *Revista de Agricultura Neotropical*, 6(1), 66-73. DOI: https://periodicosonline.uems.br/index.php/agrineo/article/view/2648/2754

Costa, E., Dias, J. G., Lopes, K. G., Silva, B. F. F. S., & Cardoso, E. D., (2015). Telas de sombreamento e substratos na produção de mudas de *Dipteryx alata* Vog. *Floresta e Ambiente*, 22 (3), 416-425. DOI: http://dx.doi.org/10.1590/2179-8087.071714

Diniz, J. M. T., Dantas, R. T., & Fideles, F. J., (2014). Spatialtemporal variability of temperature and soil thermal diffusivity from Lagoa Seca-PB. *Revista Ambiente & Água*, 9(4), 722-736. DOI: http://dx.doi.org/10.4136/1980-993X

Dionisio, L. F. S., Auca, E. C., Bardales-Lozano, R. M., Schwartz, G., & Corvera-Gomringer, R., (2019a). Production of *Bertholletia excelsa* Humb. & Bonpl.,(Lecythidaceae) seedlings in microenvironments under different substrates. *Revista Brasileira de Ciências Agrárias*, 14(3), e5847. DOI: https://doi.org/10.5039/agraria.v14i3a5847

Dionisio, L. F. S., Cusi, E. A., Schwartz, G., Bardales Lozano, R., Agurto, J. J. M., & Corvera Gomringer, R., (2019b). Seedling production of *Bertholletia excelsa* in response to seed origin and position inside fruit. *Revista Brasileira de Ciências Agrárias*, 14(3), e5662. DOI: https://doi.org/10.5039/agraria.v14i3a5662

Dionisio, L. F. S., Cusi, E. A., Milhomem, C. A., Schwartz, G., Agurto, J. J. M., & Corvera Gomringer, R., (2020). *Bertholletia excelsa*: growth and quality of seedlings produced under different environmental conditions. *Brazilian Journal of Development*, 6(6), 38589-38603. DOI: https://doi.org/10.34117/bjdv6n6-414

Dranski, J. A. L., Malavasi, U. C., & Malavasi, M. M., (2015). Relationship between lignin content and quality of *Pinus taeda* seedlings. *Revista Árvore*, 39(5), 905-913. DOI: https://doi.org/10.1590/0100-67622015000500013

Lima Filho, P., Leles, P. S. S., Abreu, A. H. M., Silva, E. V., & Fonseca, A. C., (2019). Produção de mudas de *Ceiba speciosa* em diferentes volumes de tubetes utilizando o biossólido como substrato. *Ciência Florestal*, 29(1), 27-39. DOI: https://doi.org/10.5902/1980509819340

Marana, J. P., Miglioranza, É., & Fonseca, P. É., (2015). Qualidade de mudas de jaracatiá submetidas a diferentes períodos de sombreamento em viveiro. *Revista Árvore*, 39(2), 275-282. DOI: https://doi.org/10.1590/0100-67622015000200007

Marques, A. R. F., Oliveira, V. S., Boligon, A. A. & Vestena, S., (2018). Produção e qualidade de mudas de *Psidium cattleianum* var. *cattleianum* Sabine (Myrtaceae) em diferentes substratos. *Acta Biológica Catarinense*, 5(1), 5-13. DOI: http://dx.doi.org/10.21726/abc.v5i1.374

Mori, S. A. & Prance, G. T., (1990). Taxonomy, ecology, and economy botany of the Brazil nut (*Bertholletia excelsa* Humb. and Bonpl.: Lecythidaceae). *Advances in Economic Botany*, 8, 130-150. DOI: https://www.jstor.org/stable/43927571

Nascimento, M., Andrade, K. P., Freitas, R. A., Silva, G. O., & Boiteux, L. S., (2016). Germinação de sementes de tomateiro em diferentes temperaturas: Variabilidade fenotípica e heterose. *Horticultura Brasileira*, 34(2), 216-222. DOI: http://dx.doi.org/10.1590/S0102-053620160000200011

Nogueira, I. M. S., Lahr, F. A. R., & Giacon, V. M., (2018). Desenvolvimento e caracterização de painéis de partículas aglomeradas utilizando o resíduo do ouriço da Castanha-do-Brasil (*Bertholletia excelsa*) e resina poliuretana derivada do óleo da mamona. *Revista Matéria*, 23(1), e-11985. DOI: http://dx.doi.org/10.1590/s1517-707620170001.0321

Pinho, E. K. C., Lopes, A. N. K., Costa, A. C., Silva, A. B. V., Vilar, F. C. M., & Reis, R. D. G. E., (2018). Substratos e tamanhos de recipiente na produção de mudas de baruzeiro (*Dipteryx alata* Vog.). *Revista Ciência Agrícola*, 16(1), 11-19. DOI: http://www.seer.ufal.br/index.php/revistacienciaagricola/article/view/4303

Reyes, J. T. S., Flores, H. J. M., Pérez, D. C. M. A., Sánchez, A. R., & Ramos, J. H., (2014). Calidad de planta de tres especies de pino en el vivero "Morelia", estado de Michoacán. *Revista Mexicana de Ciencias Forestales*, 5(26), 98-111. DOI: http://www.scielo.org.mx/pdf/remcf/v5n26/v5n26a8.pdf

Ribeiro, R. R., Brun, F. G. K., Brun, E. J., Mezzalira, C. C., Frigotto, T., Navroski, M. C., & Souza, M. A. M., (2018). Desenvolvimento e nutrição de mudas de acácia-negra (*Acacia mearnsii* de Wild. De Wild.) em substratos a base de cama de aviário. *Revista de Ciências Agroveterinárias*, 17(1), 36-44. DOI: http://dx.doi.org/10.5965/223811711712018036

Santos, J. U. M., Bastos, M. N. C., Gurgel, E. S. C., & Carvalho, A. C. M., (2006). *Bertholletia excelsa* Humboldt & Bonpland (Lecythidaceae): aspectos morfológicos do fruto, da semente e da plântula. *Boletim do Museu Paraense Emílio Goeldi: Ciências Naturais*, 1(2), 103-112. DOI: https://repositorio.museu-goeldi.br/handle/mgoeldi/193

Scoles, R., Canto, M. S., Almeida, R. G., & Vieira, D. P., (2016). Sobrevivência e frutificação de *Bertholletia excelsa* Bonpl. em áreas desmatadas em Oriximiná, Pará. *Floresta e Ambiente*, 23(4), 555-564. DOI: https://doi.org/10.1590/2179-8087.132015

Scoles, R. & Gribel, R., (2011). Population structure of Brazil nut (*Bertholletia excelsa*, Lecythidaceae) stands in two areas with different occupation histories in the Brazilian Amazon. *Human Ecology*, 39(4), 455-464. DOI: https://doi.org/10.1007/s10745-011-9412-0

Siqueira, D. P., Carvalho, G. C. M. W., Barroso, D. G., & Marciano, C. R., (2018). Lodo de esgoto tratado na composição de substrato para produção de mudas de *Lafoensia glyptocarpa*. *Floresta*, 48(2), 277-284. DOI: https://doi.org/10.5380/rf.v48i2.55795

Souza, J. C., Pedrozo, C., Pedrozo, C. Â., Silva, K. D., Oliveira, V. X. A., & Alencar, A. D. S., (2019). Ambientes para a produção de mudas e nodulação por rizóbios em *Tachigali vulgaris*. *Ciência Florestal*, 29(1), 116-129. DOI: https://doi.org/10.5902/1980509827955

Wadt, L. H. O., Faustino, C. L., Staudhammer, C. L., Kainer, K. A., & Evangelista, J. S., (2018). Primary and secondary dispersal of *Bertholletia excelsa*: Implications for sustainable harvests. *Forest Ecology and Management*, 415, 98-105. DOI: https://doi.org/10.1016/j.foreco.2018.02.014

Kashe K., Teketay, D., Mmusi, M., Kemosedile, T., Khululo Galelebalwee M. (2022): Assessment of diversity and composition of tree species in residential areas of Chobe District, northern Botswana. Agriculture and Forestry, 68 (1): 233-245. doi:10.17707/AgricultForest.68.1.15

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ASSESSMENT OF DIVERSITY AND COMPOSITION OF TREE SPECIES IN RESIDENTIAL AREAS OF CHOBE DISTRICT, NORTHERN BOTSWANA

SUMMARY

A vegetation survey was conducted in Chobe district to document tree species in residential areas. The survey resulted in 44 tree species, representing 44 genera and 22 families across the nine study villages. The diversity and evenness ranged from 2.47–3.15 and 0.77–0.90, respectively. An average of 19, 7 and 5 native, exotic and alien tree species respectively, were recorded. The most frequent native tree species, e.g. *Baikiaea plurijuga,* in the residential areas was also the dominant species in the neighbouring woodlands, suggesting that the species was retained when the woodlands were converted to residential use. The indigenous fruit bearing tree species, namely *Berchemia discolor* and *Sclerocarya birrea*, and the exotic fruit-bearing tree species, namely *Carica papaya* and *Mangifera indica,* were dominant in most residential areas, signifying their contribution to household food security. Invasive alien tree species, *Jatropha curcas* and *Leucaena leucocephala* were most frequent and, therefore, need continuous monitoring to prevent their spread into natural ecosystems. The study recommends raising of public awareness about invasiveness of alien invasive tree species.

Keywords: Native species; exotic species; alien species; invasive species; ecosystem services

INTRODUCTION

Tree species provide ecosystem services such as provisioning services (e.g. fuel-wood), cultural servies (e.g. spiritual service) and regulating services (e.g. climate regulation). In rural areas, they are essential in provision of firewood

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and building materials for the majority of the poor and vulnerable communities (de Neergaard et al., 2005).

In addition, trees provide food, shelter and ecological benefits to humans (Dickie et al., 2014). For instance, native species Sclerocarya birrea (A.Rich.) Hochst. and Berchemia discolor (Klotzsch) Hemsl. provide food for humans and exotic nitrogen fixing trees Prosopis species and Leucaena leucocephala provide fodder for livestock. In urban areas, trees provide a suite of benefits that enhance environmental quality and residential quality of life (Gerstenberg & Hofmann, 2016). They provide shade and reduce air temperature, sequester carbon to mitigate greenhouse gas emissions and purify atmospheric air (Nowak et al., 2013). The role of trees become even more important in the face of the negative impact of climate change.

Alien trees are a double-edged sword. They provide ecosystem services and, at the same time, harm biodiversity and ecosystem functioning (van Wilgen & Richardson, 2014), leading to a reduction in ecosystem services. The multiple benefits of trees also present a challenge for their control, as eradication programmes sometimes elicit emotional responses (Dickie et al., 2014).

A number of tree species were intentionally introduced to Botswana to stabilize sand dunes and restore vegetation in the arid southwest part of the country. These include species, such as Prosopis spp. (Fabaceae), Eucalyptus camaldulensis Dehnh (Myrtaceae), E. sideroxylon, Leucaena leucocephala (Lam) de Wit (Fabaceae), Acacia saligna (Labill.) H.L. Wendl. (Fabaceae) and Casuarina cunninghamiana Miq. (Casuarinaceae) (Lepetu, 1998). Other species such as Schinus molle L., Melia azedarach L., Ailanthus altissima (Mill.) Swingle, Spathodea campanulata P. Beauv, Tecoma stans (L.) Juss. ex Humb., Bonpl. & Kunth and Senna spectabilis (DC.) H. S. Irwin & Barneby, are present as garden or street trees.

Alien invasive plant species have been extensively studied in neighbouring South Africa (Keet et al., 2022; Potgieter et al., 2019; Shackleton et al., 2019). Despite evidence of exotic tree species in Botswana, an inventory documenting tree species in residential areas of various districts is surprisingly scarce. The only study to date was by Mafokate et al. (2013) who surveyed exotic woody plant species in the city of Gaborone in southern Botswana. Thus, similar studies are needed in other parts of the country to develop a national inventory documenting tree species in residential areas. Such inventory would be useful for effective conservation and management of tree species in residential areas.

The aim of the study was to assess the diversity and composition of tree species in residential areas of Chobe District, northern Botswana. The specific objectives were to (i) assess diversity and evenness and (ii) determine the composition of tree species in residential areas.

MATERIAL AND METHODS

Study area

The study was conducted in Chobe District (Figure 1), in the villages of Pandamatenga (pop. est. 1798), Lesoma (pop. est. 613), Kasane (pop. est 9008) and Kazungula (pop. est 4133), and the western villages namely Mabele (pop. est. 773), Kavimba (pop. est. 549), Kachikau (pop. est. 1356), Satau (pop. est. 605) and Parakarungu (pop. est. 845) (Statistics Botswana, 2011). The western villages are referred to as Chobe Enclave and covers 1690 km2 (Jones, 2002). Kasane township is the district's headquarter and a gateway to tourism in northern Botswana. It is the main government service center and houses accommodation facilities, such as hotels, lodges and guest houses. Communities in the villages of Kazungula, Kasane and western villages are settled in close proximity to Chobe River and depend on the river for resources, such as fish, farming and transportation. Pandamatenga is about 100 km south of Kasane and due to its fertile pellic vertisols (Pardo et al., 2003), it has been designated for commercial arable farming to promote government's policy of national food self-sufficiency. Chobe District receives an annual rainfall of about 640 mm occurring in the hot summer months from October to April (Botswana Meteorological Service Department unpublished data). October is usually the hottest month with a mean daily temperature of 35 oC and a mean daily minimum of 14 oC. The winter season is from the months of May to July and is dry with mean monthly temperature range of 8 - 28.5 oC. Deciduous trees start to drop their leaves in July.

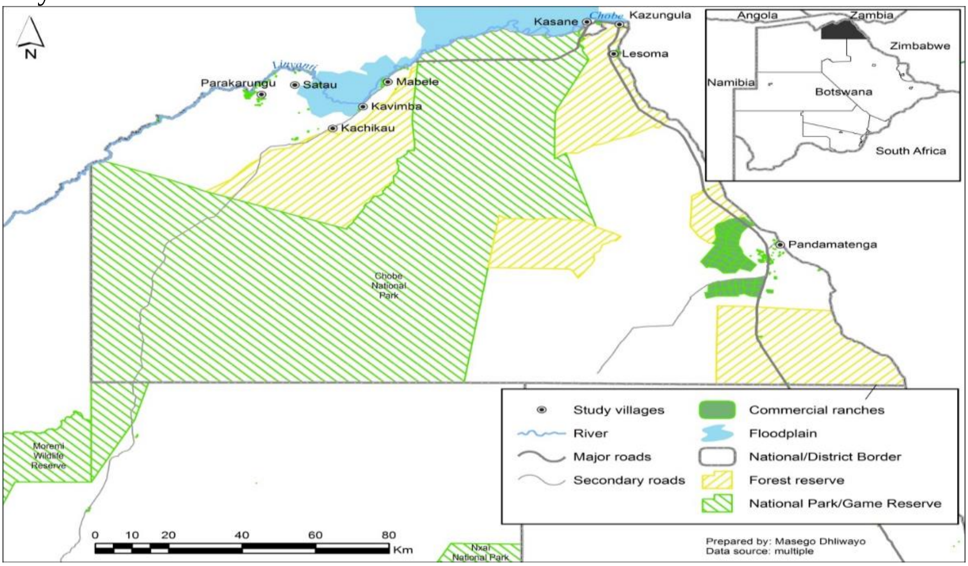

Figure 1. Map of the study area.

Data collection

The diversity of woody species was analysed using Shannon Diversity Index (H'). It is also referred to as the Shannon-Weiner or Weaver Diversity Index

(Magurran, 2004). The woody species diversity was determined by using the following formula:

$$H' = -\sum_{i=1}^{S} Pi \ln Pi$$

Where, H' = Shannon index, S = species richness, P_i = proportion of S made up of the i^{th} species (relative abundance).

Evenness or equitability, measure similarity of the abundance of the different woody species in the different habitats and was analysed by using Shannon's Evenness. Its value ranges from 0 to 1 with 1 being complete evenness. It is calculated by the following formula:

$$J' = \frac{H'}{\ln(S)},$$

Where, J' = evenness and S = species richness.
The frequency was calculated as the proportion (%) of the number of household or residential plots in which each tree species was recorded from the total number of residential plots surveyed in each of the villages.

RESULTS

A total of 44 tree species, representing 44 genera and 22 families were recorded across the nine villages (Tables 1 and 2). Of these, 19, 7 and 5 were native, exotic and alien invasive tree species, respectively (Figure 2). Lesoma exhibited the highest richness in native tree species followed by Kasane township and Kazungula. The villages of Mabele and Satau had lowest native tree species richness. For exotic species, Kasane township was the richest with 12 species, followed by its "suburb" Kazungula with nine species, and Lesoma and Kachikau with eight each. The other villages recorded seven or less number of species with Mabele and Kavimba being the least with five species each. Similarly, Kasane township and Kazungula, recorded most alien invasive tree species together with Satau. The villages of Lesoma and Satau recorded the lowest with 3 species each.

Overall, Fabaceae was the richest family with seven species followed by Combretaceae with three, and Anacardiaceae and Arecaceae with two each. Fabaceae exhibited the highest richness in tree species in Lesoma, Kasane and Kachikau followed by Kazungula and Kavimba. Fabaceae was least diverse in Pandamatenga, Satau and Parakarungu. Combretaceae was the richest family in Lesoma followed by Pandamatenga and Kavimba. Combretaceae had lowest species richness in Mabele and Satau where it was represented by only one species. However, the proportion of Combretaceae family in Kasane township (5%) and Kazungula village (7.7%) was low compared with Pandamatenga (15.4%) and Lesoma (14.3%) (Table 3).

Table 1. List of tree species with their frequencies (%) recorded in residential areas of the study villages

Species	Village*								
	PND	**LSM**	**KZN**	**KSN**	**MBL**	**KVB**	**KCH**	**STU**	**PRK**
Vachellia erioloba E.Mey.	-	1	1	0.3	-	4.3	-	-	19
Vachellia kirkii Oliv.	-	3.1	-	-	2	4.3	1.4	2.8	-
Senegalia nigrescens Oliv.	6.6	3.1	2.9	1.3	8	4.3	2.8	-	-
Vachellia tortilis (Forssk.)	1.5	37.5	17.3	1.9	8	8.7	8.5	-	1.7
Adansonia digitata L.	2.9	3.1	8.7	2.2	-	4.3	4.2	5.6	
Ailanthus altissima (Mill.)	-	-	3.8	1	8	2.2	7	36.1	1.7
Albizia versicolor Welw. Ex Oliv.	-	-	-	-	-	-	-	11.1	3.4
Azanza garckeana (F.Hoffm.) Exell & Hillc.	6.6	15.6	6.7	3.5	4	-	-	-	1.7
Baikiaea plurijuga Harms	-	2.1	52.9	31.4	-	45.7	31	-	-
Baphia massaiensis Taub.	-	-	1.9	11.4	-	-	1.4	-	1.7
Berchemia discolor (Klotzsch) Hemsl.	16.2	19.8	28.8	35.2	26	37	49.3	30.6	29.3
Boscia albitrunca (Burch.) Gilg & Benedict	0.7	3.1	-	-	-	4.3	1.4		17.2
Burkea africana Hook.	-	3.1	2.9	1.6	-	-	-	-	-
Carica papaya L.	2.9	7.3	14.4	13.3	14	26.1	12.7	19.4	20.7
Citrus limon (L.) Burm.	-	-	-	0.3	-	-	-	-	-
Citrus sinensis (L.) Osbeck	-	-	-	0.3	-	-	-	-	1.7
Colophospermum mopane (Benth.) J.Léonard	61	14.6	-	11.4	46	4.3	2.8	-	-
Combretum apiculatum Sond.	8.1	16.7	-	-	-	8.7	7	-	-
Combretum hereroense Schinz	8.8	10.4	1	-	-	-	-	-	10.3
Combretum imberbe Wawra	1.5	12.5	1.9	0.6	4	2.2	-	-	
Croton megalobotrys Müll.Arg.	-	-	-		-	4.3	1.4		5.2
Cupressus macrocarpa Hartw.	-	-	-	0.6	-	-	1.4	2.8	3.4
Dichrostachys cinerea (L.) Wight & Arn.	1.5	25	4.8	2.2	-	-	-	-	-
Eucalyptus spp.	-	-	3.8	4.8	-	2.2	-	11.1	1.7
Ficus benjamina L.	-	2.1	3.8	2.2	-	-	1.4	2.8	-
Ficus sp.	-	-	-	0.3	-	-	-	-	-
Ficus sycomorus L.	-	-	1.9	1	-	-	-	2.8	-
Hyphaene petersiana Klotzsch ex Mart	2.2	12.5	17.3	7	20	15.2	15.5	41.7	37.9
Jacaranda spp.	2.9	4.2	7.7	10.5		6.5	5.6	8.3	8.6
Jatropha curcas L.	8.8	4.2	16.3	6	10	6.5	4.2	2.8	12.1
Kigelia africana (Lam.) Benth.	-	2.1	1	0.3	-	-	-	11.1	5.2
Kirkia acuminate Oliv.	33.8	7.3	1.9	2.5	30	6.5	1.4	-	-
Leucaena leucocephala (Lam.) de Wit	-	1	16.3	23.2	12	6.5	9.9	2.8	-
Mangifera indica L.	2.2	8.3	29.8	22.9	10	8.7	12.7	11.1	5.2
Melia azedarach L.	0.7	4.2	1	-	-	-	-	2.8	5.2
Moringa oleifera Lam.	2.2	4.2	9.6	4.1	4	8.7	9.9	11.1	3.4
Morus spp.	5.9	5.2	13.5	10.2	2	2.2	11.3	5.6	1.7
Musa spp.	1.5	6.3	1	0.6	8	6.5	2.8	2.8	-
Philenoptera violacea (Klotzsch) Schrire	-	3.1	3.8	-	6	4.3	5.6	5.6	8.6
Phoenix reclinata Jacq.	0.7	3.1	9.6	4.4	-	4.3	5.6	2.8	-
Psidium guajava L.	-	1	3.8	1.6	-	-	7	13.9	10.3
Sclerocarya birrea (A.Rich.) Hochst.	25	9.4	10.6	7.6	24	13	9.9	11.1	1.7
Terminalia sericea Burch. Ex DC.	2.2	14.6	2.9	5.4	-	2.2	2.8	33.3	56.9
Trichilia emetica Vahl	7.4	14.6	21.2	19.4	26	30.4	25.4	25	17.2

Myrtaceae had highest proportion in Kasane township (10%) than in any other study villages. This family was dominated by exotic fruit tree (*Psidium guajava*) and *alien invasive tree species* (*Eucalyptus* spp.) in Kasane.The diversity of tree species ranged from 2.47 in Pandamatenga to the highest of 3.15 in Lesoma (Table 3). Similarly evenness ranged from 0.77 in Pandamatenga to 0.90 in Lesoma and Mabele (Table 3).

Table 2. Proportion of tree families (%) in residential areas of the study villages.

Species	**Village***								
	PND	**LSM**	**KZN**	**KSN**	**MBL**	**KVB**	**KCH**	**STU**	**PRK**
Anacardiaceae	7.7	5.7	5.1	5.0	13.6	9.4	9.1	7.4	7.1
Arecaceae	7.7	5.7	5.1	5.0	4.5	6.3	6.1	7.4	3.6
Bignoniaceae	3.8	5.7	7.7	7.5	0	3.1	3.0	7.4	7.1
Bombacaceae	0	0	2.6	2.5	0	3.1	3.0	3.7	0
Capparaceae	3.8	2.9	0	0	0	3.1	0	0	3.6
Caricaceae	3.8	2.9	2.6	2.5	4.5	3.1	3.0	3.7	3.6
Combretaceae	15.4	14.3	7.7	5.0	4.5	12.5	6.1	3.7	10.7
Cupressaceae	0	0	0	2.5	0	0	3.0	3.7	3.6
Euphorbiaceae	3.8	2.9	2.6	2.5	4.5	6.3	6.1	3.7	7.1
Fabaceae	15.4	28.5	23.1	25.0	27.3	25.0	30.3	18.5	17.9
Kirkiaceae	3.8	2.9	2.6	2.5	4.5	3.1	3.0	0	0
Malvaceae	3.8	2.9	2.6	2.5	4.5	3.1	0	0	3.6
Meliaceae	7.7	5.8	5.1	2.5	4.5	3.1	3.0	7.4	7.1
Moraceae	3.8	5.8	10.3	5.0	4.5	3.1	6	11.1	3.6
Moringaceae	3.8	2.9	2.6	2.5	4.5	3.1	3.0	3.7	3.6
Musaceae	3.8	2.9	2.6	2.5	4.5	3.1	3	3.7	0
Myrtaceae	3.8	2.9	5.1	10.0	0	3.1	3.0	7.4	7.1
Rhamnaceae	3.8	2.9	5.1	2.5	4.5	3.1	3.0	3.7	3.6
Rutaceae	0	0	0	5.0	0	0	0	0	3.6
Sapindaceae	0	0	2.6	2.5	4.5	0	3.0	0	0
Simaroubaceae	0	0	2.6	2.5	4.5	3.1	3.0	3.7	0
Tiliaceae	3.8	2.9	2.6	2.5	0	0	0	0	0

The frequencies of tree species ranged between 0.3 and 61% across the study villages. The five most frequent tree species were native species, namely *Colophospermum mopane* (61%) in Pandamatenga, *Terminalia sericea* (56.9%) in Parakarungu, *Baikiaea plurijuga* (52.9%) in Kazungula, *Berchemia discolor* (49.3%) in Kachikau and *Hyphaene petersiana* in Satau. Fruit trees, both native and exotic species were observed in the study villages.

The exotic fruit tree species recorded in all the study villages were *Carica papaya*, *Mangifera indica*, *Morus* and *Musa* species. The two most frequent species were *M. indica* in Kazungula and *C. papaya* in Kavimba. *Berchemia discolor* and *Sclerocarya birrea*, indigenous fruit tree species, were observed in all the study villages and were the most frequent with 49.3% in Kachikau and 25% in Pandamatenga, respectively.

Table 3. Diversity and evenness of trees species in residential areas of the study villages

Village	**Diversity (*H’*)**	**Evenness (*J’*)**
Pandamatenga	2.47	0.77
Lesoma	3.15	0.90
Kazungula	3.03	0.86
Kasane	2.94	0.82
Mabele	2.69	0.90
Kavimba	2.93	0.87
Kachikau	2.92	0.86
Satau	2.88	0.88
Parakarungu	2.77	0.84

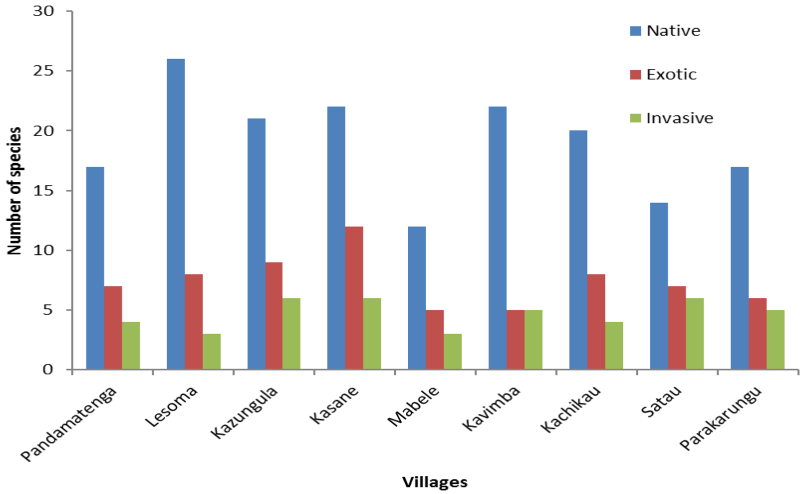

Figure 2. Number of tree species by types in the study villages in Chobe District.

Invasive alien tree species at different stages of infestation were recorded in different areas. *Ailanthus altissima*, *Jatropha curcas* and *Leucaena leucocephala* were at advanced stage of infestation and were recorded in almost all the study villages. These invasive species were most frequent in Satau, Kazungula and Kasane, with frequencies of 36.1, 16.3 and 23.2%, respectively. *Eucalyptus* spp. and *Melia azedarach* were at low stages of infestation and were only observed in half of the study villages at low frequencies.

DISCUSSION

The results demonstrated that the residential areas of the villages in Chobe District exhibited diversity of tree species. The overall diversity of tree species is much higher in all the study villages with a range of 2.47 to 3.15, indicating high species richness. The value of H' usually falls between 1.5 and 3.5 (Magurran, 2004). This implies that the diversity of tree species in the study villages falls on the higher value of the diversity range.

The residential areas in the different study villages were dominated by species that were also the dominant species in surrounding woodlands, suggesting that they were the main species before the woodlands were converted to residential use and were retained in homestead to provide ecosystem services. It is safe to suggest that some residents retained these species for their ecosystem services, e.g. shade and food provision. *Colophospermum mopane* exhibited high frequency in Pandamatenga and Mabele. It grows in a variety of soils from heavy clay soils to sandy soils (Mapaure, 1994), and some areas on "black cotton soils" (Barbosa, 1970). It was, therefore, not surprising to observe that it was dominant in Pandamatenga, an area that has been designated for commercial arable farming owing to its fertile "black cotton soils" (Pardo et al., 2003; Abdullahi, 2004). *Baikiaea plurijuga* was the most frequent species in residential areas of Kazungula, Kasane and Kavimba. This observation is consistent with vegetation studies in the same area, which also found *B. plurijuga* to be one of the dominant species in the mixed woodlands (Ben-Shahar, 1998; Rutina et al., 2005).

The residential areas in the villages of Satau and Parakarungu were dominated by *Hyphaene petersiana*, a species usually common on islands in the floodplains (Heath & Heath, 2009). The two villages partly lie in the floodplain of Chobe River and thus explain why this species was recorded in most residential areas of these villages. The abundance of *H. petersiana* can also be attributed to its multiple uses. Its sap is a source of palm wine and young unopened leaves are used to weave baskets (Mollet et al., 2000; Babitseng & Teketay, 2013). Basketry production is an important income generating activity, particularly for women in Botswana. In addition to *H. petersiana*, *Terminalia sericea* was also dominant in Satau and Parakarungu than in any other villages. Its abundance could be partly explained by its preference to transition zone between sandy and clay soils (Tinly, 1992). Additional, *T. sericea* is common and widely distributed in southern Africa (McGaw et al., 2001; Shackleton, 2001) where it provides a suite of ecosystem services, such as fuel, timber and medicine (Van Wyk & van Wyk, 2013). It is used to treat both ethnoveterinary infections and a variety of human infections (Mongalo et al., 2016

The diversity of exotic and indigenous fruit-bearing trees in the residential areas highlights their contribution to household food security. The indigenous fruit-bearing tree species, namely *Berchemia discolor* and *Sclerocarya birrea*, were recorded in residential areas of all the study villages at higher frequencies, signifying their importance as a source of food and shade. The fruits of these two tree species are rich in vitamins C which are higher than that of the exotic species

(Chivandi et al., 2012). Exotic fruit-bearing trees were dominated by *Carica papaya* and *Mangifera indica* which were observed in residential areas of all the study villages at high frequencies. *Mangifera indica* was preferred probably for its drought tolerance (Greisbach, 2003) and ability to produce many fruits (200 - 500) per year (Jama et al., 2008). Furthermore, *M. indica* can be easily propagated vegetatively or by seed (Greisbach, 2003). Similarly, *Carica papaya* was probably favoured for its fast-growth, with the fruits produced within 10 - 14 months from germination (Silva et al., 2007). The fruit is eaten as fresh fruit and as a vegetable. It is easily propagated by seeds (Chaves-Bedoya & Nuñez, 2007). The two species are widely distributed and adapted to climatic and environmental conditions in Sub-Saharan Africa (Chivandi et al., 2012).

Invasive alien tree species *Jatropha curcas* (Negussie et al., 2013) and *Leucaena leucocephala* (Lyons & Miller, 1999) were the most frequent in residential areas of the study villages. These fast-growing trees were intentionally introduced to Botswana as candidate for biodiesel and fodder production respectively. They have since escaped their intended use and communities are now planting them for landscaping, shade and building material. If not properly managed, the two species can become invasive and invade neighbouring ecosystems. Such invasion will negatively impact ecosystem functioning and consequently, ecosystem services (Dickie et al., 2014). Invasive alien tree species were more prevalent in Kasane township and its “suburb” Kazungula. In a similar study in the Eastern Cape, South Africa, Mabusela et al. (2021) recorded more woody invasive alien species in townships than in other neighbourhoods. In Western Cape, South Africa, McLean et al. (2018) also found 50% alien invasive plant species recorded to be naturalized within town. Mafokate et al. (2013) recorded 26 alien invasive woody species in Gaborone, capital city of Botswana. This was not surprising because majority of invasive species are usually more prolific in cities because of high disturbance and habitat fragmentation that facilitate invasion process (Gaertner et al., 2016). On the contrary, the invasive and poisonous *Ailanthus altissima* (Rebbeck & Jolliff, 2018; Petruzzellis et al., 2018) was almost absent in Kasane but was the second dominant species in the rural village of Satau, where it is grown as an ornamental and shade tree. The impact of alien invasive tree species can be profoundly massive (Hejda et al., 2017), and often occupy a wide modified area (Pyšek et al., 2012), creating conditions unfavourable for some indigenous species (Richardson & Rejmánek, 2011).

CONCLUSIONS

Woody species diversity is an essential component of forest or woodland diversity and thus central to their biodiversity. The information on plant diversity is useful for planning effective conservation and management strategies. The study revealed a relatively high species and family richness of tree species in the study area. The diversity and evenness values of the villages were relatively high suggesting that most tree species were uniformly distributed across the study villages. The study also revealed that most residential areas in Choke District were dominated by native tree species, which indicates their importance in provision of ecosystem services. Such important species were probably retained when the woodlands were converted to residential use implying the importance of conserving indigenous tree species. The study also showed prevalence of alien trees species particularly in residential areas of ‘town’ and surrounding villages, indicating the need for continuous monitoring to prevent their spread into other ecosystems. The study recommends consultation with the local community to remove invasive tree species from their homesteads and replace them with indigenous tree species that will provide the same ecosystem services. The study further recommends ethnobotanical survey of woody species in the study area to generate information on how communities are using the different species.

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REFERENCES

Abdullahi, A. E. (2004). Weed survey in cotton (*Gossypium hirsutum* L.) and sunflower (*Helianthus annuus* L.) fields in the Pandamatenga plains of northeastern Botswana. *South African Journal of Plant and Soil*, *21*(1), 21–24.

Babitseng, T. M., & Teketay, D. (2013). Impact of wine tapping on the population structure and regeneration of *Hyphaene petersiana* Klotzsch ex Mart. in northern Botswana. *Ethnobotany Research and Applications*, *11*, 009–027.

Barbosa, L. A. G. (1970). Carta Fitogeographica de Angola. Institute de Investigacao Cientifica de Angola. Luanda

Ben-Shahar, R. (1998). Changes in structure of savanna woodlands in northern Botswana following the impacts of elephants and fire. *Plant Ecology*, *136*(2), 189–189.

Chaves-Bedoya, G., & Nuñez, V. (2007). A SCAR marker for the sex types determination in Colombian genotypes of *Carica papaya*. *Euphytica*, *153*(1-2), 215–220.

Chivandi, E., Davidson, B. C., & Erlwanger, K. H. (2012). Red Sour Plum (*Ximenia caffra*) Seed: A Potential Nonconventional Energy and Protein Source for Livestock Feeds. *International Journal of Agriculture & Biology*, *14*(4).

Chivandi, E., Mukonowenzou, N., Nyakudya, T., & Erlwanger, K. H. (2015). Potential of indigenous fruit-bearing trees to curb malnutrition, improve household food security, income and community health in Sub-Saharan Africa: A review. *Food Research International*, *76*, 980–985.

de Neergaard, A., Saarnak, C., Hill, T., Khanyile, M., Berzosa, A. M., & Birch-Thomsen, T. (2005). Australian wattle species in the Drakensberg region of South Africa–An invasive alien or a natural resource?. *Agricultural Systems*, *85*(3), 216–233.

Dickie, I. A., Bennett, B. M., Burrows, L. E., Nuñez, M. A., Peltzer, D. A., Porté, A.,.. & Van Wilgen, B. W. (2014). Conflicting values: ecosystem services and invasive tree management. *Biological Invasions*, *16*(3), 705–719.

Gaertner, M., Larson, B. M., Irlich, U. M., Holmes, P. M., Stafford, L., van Wilgen, B. W., & Richardson, D. M. (2016). Managing invasive species in cities: A framework from Cape Town, South Africa. *Landscape and Urban Planning*, *151*, 1–9.

Gerstenberg, T., & Hofmann, M. (2016). Perception and preference of trees: A psychological contribution to tree species selection in urban areas. *Urban Forestry & Urban Greening*, *15*, 103–111.

Heath A, Heath R (2009). Field Guide to the Plants of Northern Botswana: Including the Okavango Delta. Kew: Kew Publishing, Royal Botanic Gardens.

Hejda, M., Hanzelka, J., Kadlec, T., Štrobl, M., Pyšek, P., & Reif, J. (2017). Impacts of an invasive tree across trophic levels: Species richness, community composition and resident species’ traits. *Diversity and Distributions*, *23*(9), 997–1007.

Jama, B. A., Mohamed, A. M., Mulatya, J., & Njui, A. N. (2008). Comparing the “Big Five”: A framework for the sustainable management of indigenous fruit trees in the drylands of East and Central Africa. *Ecological Indicators*, *8*(2), 170–179.

Jones, B. T. B. (2002). Chobe Enclave, Botswana - lessons learnt from a CBNRM project; 1993–2002. Gaborone: IUCN/SNV CBNRM Support Programme.

Keet, J. H., & Richardson, D. M. (2022). A rapid survey of naturalized and invasive eucalypt species in southwestern Limpopo, South Africa. *South African Journal of Botany, 144,* 339-346.

Lepetu J (1998). Investigation on sustainability of species for sand dune stabilization in the Kalahari desert with special reference to Tsabong, Botswana. SACCAR Newsletter 42: 28–32.

Lyons EE, Miller SE (Eds) (1999) Invasive Species in Eastern Africa: Proceedings of a Workshop held at ICIPE, July 5–6, 1999.

Mabusela, A., Shackleton, C. M., & Gwedla, N. (2021). The distribution of selected woody invasive alien species in small towns in the Eastern Cape, South Africa. *South African Journal of Botany*, *141*, 290-295.

Mafokate D, Mathowa T, Mojeremane W (2013) A survey of the exotic woody plant species in the city of Gaborone, Botswana. *International Journal of Research in Agriculture and Food Sciences*, *1*, 2.

Magurran, A. E. (2004). *Measuring biological diversity*. Malden and Oxford: Blackwell Publishing.

Mapaure, I. (1994). The distribution of *Colophospermum mopane* (Leguminosae-caesalpinioideae) in Africa. *Kirkia*, 1–5.

McGaw, L. J., Rabe, T., Sparg, S. G., Jäger, A. K., Eloff, J. N., & Van Staden, J. (2001). An investigation on the biological activity of *Combretum* species. *Journal of Ethnopharmacology*, *75*(1), 45–50.

McLean, P., Wilson, J. R., Gaertner, M., Kritzinger-Klopper, S., & Richardson, D. M. (2018). The distribution and status of alien plants in a small South African town. *South African Journal of Botany, 117,* 71-78.

Mollet, M., Herzog, F., Behi, Y. E. N., & Farah, Z. (2000). Sustainable exploitation of *Borassus aethiopum*, *Elaeis guineensis* and *Raphia hookeri* for the extraction of palm wine in Côte d'Ivoire. *Environment, Development and Sustainability*, *2*(1), 45–59.

Mongalo, N. I., et al. "Ethnobotany, phytochemistry, toxicology and pharmacological properties of *Terminalia* sericea Burch. ex DC.(Combretaceae)–A review. *Journal of Ethnopharmacology*, *194,* 789–802.

Negussie, A., Achten, W. M., Aerts, R., Norgrove, L., Sinkala, T., Hermy, M., & Muys, B. (2013). Invasiveness risk of the tropical biofuel crop *Jatropha curcas* L. into adjacent land use systems: from the rumors to the experimental facts. *GCB Bioenergy*, *5*(4), 419–430.

Nowak, D. J., Greenfield, E. J., Hoehn, R. E., & Lapoint, E. (2013). Carbon storage and sequestration by trees in urban and community areas of the United States. *Environmental Pollution*, *178*, 229–236.

Nowak, D. J., Hirabayashi, S., Bodine, A., & Greenfield, E. (2014). Tree and forest effects on air quality and human health in the United States. *Environmental Pollution*, *193*, 119–129.

Petruzzellis, F., Nardini, A., Savi, T., Tonet, V., Castello, M., & Bacaro, G. (2018). Less safety for more efficiency: water relations and hydraulics of the invasive tree *Ailanthus altissima* (Mill.) Swingle compared with native *Fraxinus ornus* L. *Tree Physiology*. https://doi.org/10.1093/treephys/tpy076 2018 Jul 3.

Pardo, M. T., Ristori, G., D'ACQUI, L. P., & Almendros, G. (2003). An assessment of soil fertility and agronomic constraints in southern African savannas: A case study of the Pandamatenga area, Botswana. *South African Geographical Journal*, *85*(1), 35–41.

Potgieter, L. J., Gaertner, M., O'Farrell, P. J., & Richardson, D. M. (2019). Perceptions of impact: invasive alien plants in the urban environment. *Journal of Environmental Management*, *229*, 76-87.

Pyšek, P., Jarošík, V., Hulme, P. E., Pergl, J., Hejda, M., Schaffner, U., & Vilà, M. (2012). A global assessment of invasive plant impacts on resident species, communities and ecosystems: the interaction of impact measures, invading species' traits and environment. *Global Change Biology*, *18*(5), 1725-1737.

Rebbeck, J., & Jolliff, J. (2018). How long do seeds of the invasive tree, Ailanthus altissima remain viable?. *Forest Ecology and Management*, *429*, 175–179.

Rejmánek, M., & Richardson, D. M. (2013). Trees and shrubs as invasive alien species–2013 update of the global database. *Diversity and Distributions*, *19*(8), 1093–1094.

Rejmánek, M., Huntley, B. J., Le Roux, J. J., & Richardson, D. M. (2017). A rapid survey of the invasive plant species in western Angola. *African Journal of Ecology*, *55*(1), 56–69.

Richardson, D. M., & Rejmánek, M. (2011). Trees and shrubs as invasive alien species–a global review. *Diversity and Distributions*, *17*(5), 788–809.

Richardson, D. M., Pyˇsek, P., & Carlton, J. T. (2011). A compendium of essentialconcepts and terminology in invasion ecology. In D. M. Richardson (Ed.), Fiftyyears of invasion ecology. The legacy of Charles Elton. Oxford: Wiley-Blackwell,pp. 409–435.

Rutina, L. P., Moe, S. R., & Swenson, J. E. (2005). Elephant *Loxodonta africana* driven woodland conversion to shrubland improves dry-season browse availability for impalas *Aepyceros melampus*. *Wildlife Biology*, *11*(3), 207–213.

Setshogo MP (2002) Common Names of Some Flowering Plants of Botswana. FAO, Rome, Italy.

Setshogo MP (2005) Preliminary Checklist of the Plants of Botswana. Southern African Botanical Diversity Network (SABONET), Pretoria, South Africa.

Setshogo MP, Venter F (2003) Trees of Botswana: Names and Distribution. Southern African Botanical Diversity Network (SABONET), Pretoria, South Africa.

Shackleton, R. T., Shackleton, C. M., & Kull, C. A. (2019). The role of invasive alien species in shaping local livelihoods and human well-being: A review. *Journal of Environmental Management, 229,* 145-157.

Shackleton, C. M. (2001). Managing regrowth of an indigenous savanna tree species (*Terminalia sericea*) for fuelwood: the influence of stump dimensions and post-harvest coppice pruning. *Biomass and Bioenergy*, *20*(4), 261–270.

Silva, J. D., Rashid, Z., Nhut, D. T., Sivakumar, D., Gera, A., Souza, M. T., & Tennant, P. (2007). Papaya (*Carica papaya* L.) biology and biotechnology. *Tree and Forestry Science and Biotechnology*, *1*(1), 47–73.

Statistics Botswana. (2011). Chobe sub-district population and housing census 2011: Selected indicators for villages and localities. Gaborone: Statistics Botswana.

van Wilgen, B. W., & Richardson, D. M. (2014). Challenges and trade-offs in the management of invasive alien trees. *Biological Invasions*, *16*(3), 721–734.

Van Wyk B, van Wyk P (2013). Field Guide to the Trees of Southern Africa Second ed. Struik Nature Publishers, Cape Town, pp 202.

Rokvić Knežić, G., Đurić, J., Drinić, Lj. (2022): Agritourism as an opportunity for rural development of Prnjavor municipality. Agriculture and Forestry, 68 (1): 247-260. doi:10.17707/AgricultForest.68.1.16

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AGRITOURISM AS AN OPPORTUNITY FOR RURAL DEVELOPMENT OF PRNJAVOR MUNICIPALITY

SUMMARY

The research aim of this paper was to identify the potential of the development of rural and agritourism in the municipality of Prnjavor. The basic methods used during the research ware survey, tourist potential mapping, mapping of tourist actors and statistical methods. The aim was to identify tangible and intangible capital, but also to find out the views of the local population on rural tourism. The results of the research showed that the municipality of Prnjavor has the potential to engage in rural tourism, ie agritourism. The awareness of the population, however is not developed when it comes to rural tourism and the opportunities it provides. The recomendation of the research for local authorities is the re-formation of a tourist organization, which would promote the tourist offer of the municipality of Prnjavor and thus attract a larger number of tourists.

Keywords: agritourism, development, potentials, local community

INTRODUCTION

Rural tourism is most often defined as tourism that takes place in rural areas. It is related to the natural resources of a certain place and includes a large number of activities, such as hiking, hunting and fishing, swimming, cycling, participation in farm work and other activities. Specific characteristic of rural tourism is that there is an interaction between tourists and hosts. Different definitions of rural areas affect different definitions of rural tourism.

Rural tourism can be a complementary activity, but it can also be a professional activity in rural areas (Baćac, 2011). A narrower term than rural tourism is village tourism, which is related to the ambience of the village and various activities, such as: agriculture, gastronomy, various events and manifestations.

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Agrotourism is a narrower term than village tourism and represents an additional activity on an agricultural farm that is engaged in a certain type of agricultural production.

Šehić-Kršlak (2018) states that the concept of rural tourism refers to a form of development policy that seeks to meet the economic, social and environmental needs of society in terms of short-term, medium-term and, above all, long-term well-being.

Rural tourism takes place in the countryside, and agritourism is rural tourism that takes place on farms. Lane et al. (2013) in their paper state the reasons why this definition is inadequate:

1. Urban or resort tourism is not limited only to urban areas but through excursions, employment and shopping, it also spreads to rural areas,
2. Rural areas are difficult to define in themselves and the criteria for determining them vary greatly from nation to nation,
3. Not every tourism that takes place in rural areas is strictly "rural" - it can be urban in shape, and completely located in a rural area.

Brščić et al. (2010) define agritourism as a land-based activity, shows growth and contributes to rural development and depopulation reduction by attracting and developing new services in rural areas, opening employment opportunities, creating new infrastructure and the possibility of achieving additional sources of income.

Some authors define agrotourism as a type of tourist rural family farm in which the main activity (activity) is agricultural production, while tourist accommodation and catering services are an additional activity (Baćac, 2011). In addition to the basic catering services of accommodation and food, other tourist services (activities, service packages) can be organized on the farm, which aim to provide guests with the possibility of active vacation, ie to make the most of the tourist potential of the farm.

One of the characteristics of agritourism is that it provides tourists with the opportunity to get acquainted with the life and work of the population in rural areas, as well as their customs and culture. It is very important that the development of rural or agrotourism preserves natural potentials and uses them rationally, so that there are no negative consequences for rural areas. According to Despotović the benefits of the local community from agri-tourism development include better social infrastructure (schools, libraries, health care institutions, etc.). Furthermore, agritourism initiates better valorisation of natural resources of rural communities (Despotović et all., 2017).

The aim of this paper is to identify tangible and intangible capital, and above all the natural potential and cultural and natural heritage for the development of rural tourism in the municipality of Prnjavor, but also to find out the views of the local population on rural tourism.

In the introductory part, the theoretical setting of the problem of valorization of territorial capital is explained and the link is given to the territorial capital of the municipality of Prnjavor, and its tourist potentials. The results of the applied methodology for assessing the tourist value of internal and external factors, as well as the total tourist value of the destination are presented and discussed in the context of the case study of Prnjavor municipality. The second part of the paper presents an analysis of the attitudes of potential providers of rural tourism in order to assess the level of awareness of the benefits of rural tourism and agritourism. In conclusion, the results of the research are summarized and recommendations are given for improving the tourist value of the destination.

MATERIAL AND METHODS

The material for the preparation of this paper consists of data obtained by various research methods, as well as from the collected literature. The following methods were used in this paper:

-Description method used to describe the existing available resources in the municipality, ie mapped attractors during the research;

-Mapping of potentials or attractors in the municipality included the identification of all natural and cultural attractions (natural beauties, architecture, customs, events, etc.),

-Mapping of actors in the field of rural tourism in the municipality included the identification of existing and potential actors, ie agricultural farms and rural households, but also representatives of the public and civil sector who are active or potentially active in the field of tourism and rural tourism;

-Statistical methods, ie the method of descriptive statistics for data processing, their presentation using graphs and tables.

The survey was conducted on the case study of the municipality of Prnjavor. The survey included following respondents: 170 agricultural holdings and rural households, as existing and potential service providers, Mountain association "Step higher", Women's Association "Tkanica" and Department for Local and Economic Development and Social Activities of Prnjavor municipality. In this part of the research, a structured questionnaire was used, which includes three chapters, which actually refer to the set of hypotheses:

-hypotesis 1. The municipality of Prnjavor has the potential to engage in agritourism;

-hypotesis 2. In the area of the municipality of Prnjavor, there is a possibility of connecting agrotourism with agriculture;

-hypotesis 3. Actors in the field of rural tourism in the municipality of Prnjavor are aware of the advantages and limitations for the development of rural tourism.

RESULTS AND DISCUSSION

Bosnia and Herzegovina is a predominantly rural country with 57.3% of the rural population according to the latest census, and with an average population density of 68.9 inhabitants per km2 (BiH Agency for Statistics, 2013). The total area of Bosnia and Herzegovina is 51,222.84 km2, and the size of the rural territory is not accurately determined due to the fact that BiH does not have an established official definition for the delimitation of rural areas. Out of a total of 6141 settlements, 6020 of them are rural, so it can be assumed that most of the territory of BiH is rural. The provision of accommodation services in rural households falls into the group of "resorts and similar facilities for short vacations" which participate with 1.6% in the total overnight stays of tourists in BiH (Agency for Statistics, 2021). An entity-level analysis for Republika Srpska shows that the share of rural accommodation in total private accommodation is 10% and rose from 12 bed units in 2011 to 490 bed units in 2020 (Agencies for intermediary, information and financial affairs, 2020). Tourism is one of the three major sectors in rural areas in EU, together with agriculture and forestry. According to Eurogites, the offer within rural tourism in Europe is about 500,000 units, 5-6.5 million beds, of which 20% are units categorized as agritourism (Eurogites, 2016). Although the number of accommodation capacities in the countryside is constantly increasing, it is still not at a satisfactory level. The reason for that is the low level of awareness of the local population about the place and role of tourism in sustainable development, the lack of tradition, the rather low level of infrastructure and superstructure, the modest tourist product and poor marketing (Ministry of trade and tourism).

The municipality of Prnjavor is located in the northwestern part of Bosnia and Herzegovina, the entity of Republika Srpska, in the part that gravitates towards Banja Luka. Administratively, the territory of the Municipality covers an area of about 630 km2 or 63,000 ha. The city area of Prnjavor covers an area of about 5.5 km^2. The censuses conducted so far have recorded that a multinational population (19 nations and nationalities) lived on the territory of the Municipality, and still lives today. Due to all the above, the name "Little Europe" can be rightly said and accepted for the area of the municipality of Prnjavor (Mandić, 2019). In the last twenty years, there have been significant changes related to the population of the municipality of Prnjavor, as shown by the preliminary results of the 2013 census, according to which a total of 38,399 people were registered in 12,220 households. Available information indicates that the largest number of inhabitants in the municipality live in rural settlements, but recently there has been a noticeable trend of demographic depopulation, especially settlements further away from the municipal center. Of the total area on which the municipality of Prnjavor is spread, agricultural land covers about 68.7%. According to the degree of importance for the development of the local economy, agricultural activity is dominant source of income.

The municipality has favorable natural conditions for the development of agricultural production, mainly livestock and farming, which are precisely the most developed branches of agriculture in the municipality of Prnjavor.

It is estimated that close to 30,000 people live in the rural part of the municipality. According to the Farm Register data, over 1,700 agricultural farms are registered in the municipality.

The municipality of Prnjavor has the potential to engage in agritourism. This hypothesis was confirmed through the mapping of natural and cultural

attractions in the municipality of Prnjavor, as well as through the evaluations of the survey results.
All surveyed groups, ie agricultural holdings, associations/societies and the Department for Local and Economic Development and Social Activities evaluated the supply factors (natural and social attractions, traffic connections, supply in rural tourism, tourist mediation and support in the municipality). The highest score of 4.7, is recored for the landscape and the connection of the municipality with the highways, while the lowest score of 1.8 is given to the existence of a tourist organization and association.

Table 1. shows the average ratings of natural attractions in the municipality of Prnjavor and the average deviation of all individual ratings. The highest average grade is given to the landscape (4.7), and the lowest grade (2.7) to the development of organic agriculture. The average grade for all natural atractors is 3.7. This corresponds with another research supported by SWG for the regiona of South East Europe, where the average assessment of natural attractors is 3.5, with small variations around this value among the countries/territories. (SWG, 2020). As the most valuable natural attractions, most respondents stated the following: Banja Kulaši, Ljubić Mountain, Lake Drenova and Stud farm Vučijak.

Table 1. Assessment of natural attractions in the municipality

Natural attractions (mean value of rated elements of natural attraction)	**Average grade**	**Standard deviation**
climate	4,1	0,80
hydrographic elements (rivers, lakes)	3,9	0,77
landscape	4,7	0,63
flora and fauna	4,3	0,67
natural rarities	2,8	1,34
developed agriculture	3,9	0,99
developed ecological (organic) agriculture	2,7	1,05

Table 2. shows the average ratings of social attractions in the municipality of Prnjavor, as well as the average deviation of all individual ratings. The best rated, with an average score of 4.2, is "Expressed hospitality and positive attitudes of the local population towards tourists", while "the rural lifestyle" is rated with an average score of 3.3. The average numerical evaluation of cultural attractors in SWG study is 3.7 wich is exact the same as in the case of Prnjavor municipality (SWG, 2020). Respondents listed the following as the most valuable social attractions: Log cabin church, Stuplje Monastery and Festival of National Minorities.

The traffic connection with the municipality and within the municipality was rated with high scores. Road infrastructure in rural areas has the lowest average score of 3.5, which is actually one of the problems in rural areas. The connection of the municipality with highways was rated the best, with an average grade of 4.7. The average grade is 4.1 and it is above average evaluated for South East Europe countries where only 2.4 grade is evaluated (SWG, 2020).

Table 2. Assessment of social attractions in the municipality

Social attractions (mean value of rated elements of social attractiveness)	**Average grade**	**Stanadard deviation**
rural heritage (traditional architecture, history, castles, churches, villages)	3,7	0,89
rural way of life (local events, gastronomy, agritourism, traditional music)	3,3	1,07
pronounced hospitality and positive attitudes of the local population towards tourists	4,2	0,84
existence of conditions for recreation, leisure and sightseeing	3,6	1,08

According to research results the current offer in rural tourism is law, with "the existence of accommodation facilities in rural areas" rated the lowest, only 2.3 points. Also, offer of catering services, offer of rural products and activities in rural areas was rated very low. On the other side safety and free movement of tourist is appreciated as very good. The average grade assessed is 3.1 wich is lower in comperisson with SWG studies, where these man-made attraction have been rated with 3.5 (SWG, 2020).

Table 3. Assessment of traffic connection of the Prnjavor Municipality

Traffic connection with the municipality and within the municipality (average value of the assessed elements of natural attractiveness)	**Average grade**	**Standard deviation**
road infrastructure in rural areas	3,5	0,98
connection of the municipality with highways	4,7	0,57
close to the airport	4	1,08
proximity to major regional centers	4,2	0,68

Together with law offer, also tourist mediation and support was rated quite law. Such low grades were caused by the non-existence of a tourist organization in the municipality, as well as poor information of the population about the very concept of rural tourism, as well as the state of tourism in the municipality. The average grade of these factors is only 2.05 and it is again lower in comperisson with SWG study where services in general have gained 2.4 in total numerical evaluation (SWG, 2020).

According to Demirović and Radosavac (2018) rural tourism sector in Bosnia and Herzegovina has an abundance of natural resources and special attractions. However, ancillary services and facilities are very limited and this may affect the reduction of attractiveness, and the possibilities for the sector to highlight its potential.

Table 4. Offer in rural tourism in the municipality

Offer in rural tourism (average value of rated elements of natural attraction)	Average grade	Standard deviation
existence of accommodation capacities in a rural area (on farms, motels, hotels, boarding houses, camps ..)	2,3	1,24
existence of catering facilities in the rural area (restaurants)	2,9	1,29
offer of rural products (agricultural products, handicrafts, etc.)	3,4	1,19
offer of activities in rural areas (agricultural activities on the farm, horseback riding, cycling, fishing, hiking, water sports)	2,8	1,16
price level of products and services	2,8	1,37
the possibility of free and safe movement of tourists	4,5	0,74

Table 5. Tourist mediation and support in the municipality

Tourist mediation and support (mean value of assessed elements of natural attraction)	Average grade	Standard deviation
the existence of travel agencies	2,5	1,49
the existence of tourist organizations	1,8	1,54
the existence of tourist associations and unions	1,8	1,16
existence of marketing and promotional activities related to rural and agritourism	2	1,26
existence of strategies and plans for rural and agritourism development	2	1,21
existence of financial and professional support for the development of rural or agritourism	2,2	1,35

Gilbert (1992) remarks with respect to rural tourism, also stress the problem of law standard on supply side: The existing supply is sometimes lacking in basic amenities and a minimum level of comfort. Other research also point out that an assessment of the development of rural tourism during the nineties betrays a virtually complete lack of planning in both private and public action (Nacher, 1997) which implies the level of tourism development in Prnjavor municipality comparable with the situation existing in western European countries in the 90-ies. Analysts of the problem, therefore, appear to agree that this form of tourism needs to evolve, to be seen as a business and to be managed as such (Garcia and Grande, 2005). The same author considers it as essential that any action should be undertaken by private initiative, and based on market principles. According to Bojnec (Bojnec, 2010) the supply of rural- and farm-based tourism in most of the countries of Central and Eastern Europe is determined by demand-side factors, but also by entrepreneurial spirits in rural areas, farm diversification and even farm specialization into farm or agro tourism due to new marketing opportunities, farm-employment, income and similar reasons.

Demand factors in the municipality of Prnjavor

Average ratings of demand factors in the municipality of Prnjavor are shown in Table 6. The highest score of 3.9 was given to the increase in the level of education, and the lowest 2.8 to the increase of free time followed by the increase of disposable income.

Table 6. Assessment of demand factors in the municipality of Prnjavor

Demand factors in the municipality of Prnjavor	Average grade	Standard deviation
The need for a better environment	3,7	0,69
The need for authenticity	3,4	0,91
Increasing the level of education	3,9	0,87
Increasing interest in heritage	3,5	1,17
Increase in free time followed by increasing disposable income	2,8	1,50
Raising awareness of the importance of health	3,6	1,08
Better equipment for outdoor activities	2,9	1,37
Growing interest in traditional and special food types	3,7	0,83
Growing interest in environmental issues	3,4	0,97
Peace and tranquility as a motive for tourist demand	3,6	1,08
Age as a motive for demand (active aging)	3,4	0,81
The need for "real" travel (in which interaction with the local population takes place)	3,1	1,51
The rise of individualism (avoiding mass in favor of alternative tourism)	3	1,32

Agritourism or farm holidays are increasingly in demand in Europe and result in being a successful example of sustainable tourism that has gained importance over the years (Streifeneder and Dax, 2020). Based on literature review of Pesonen et all (2011) show that rural tourists have several similarities: they are most often motivated by opportunities to learn and explore nature or different cultures, participate in outdoor activities, search for peace and solitude. They may expect family togetherness, peace and quiet, friendly reception, change from routine and good food. Beautiful landscapes, opportunities for outdoor activity and hassle-free environments tend to attract rural tourists. But also differences can be found especially in relation to expectations towards farming activities, heritage or other destination attributes.

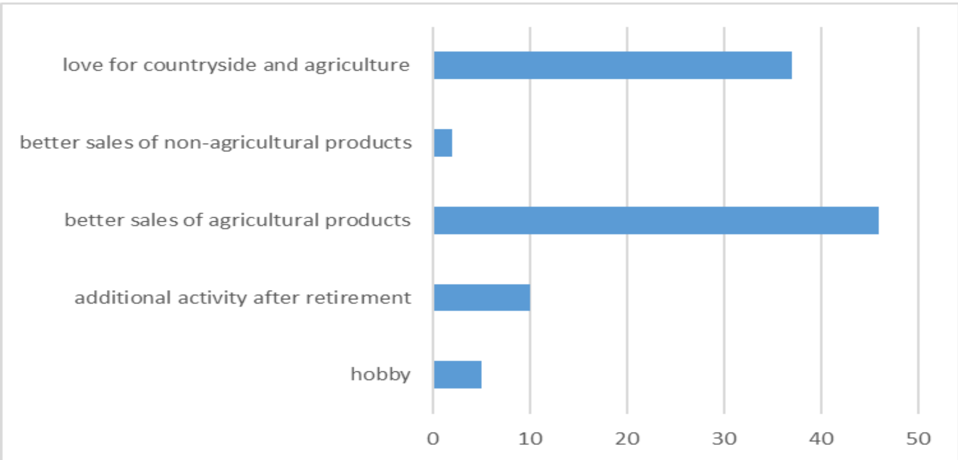
Table 7 shows the benefits of rural tourism rated by the respondents with average scores of 3.6 to 4.1. The benefits of rural tourism are numerous, and the respondents' assessments depend primarily on how familiar they are with the concept of rural tourism and their attitudes related to rural tourism.

The research of Popović and Lazarević (2021), Nagaraju and Chandrashekara (2014), Irshad (2010) but also Sharpley (2000) confirm that rural tourism is making a valuable contribution to rural economies. Its contribution can be expressed not only in financial terms, but also in terms of jobs, contributions towards funding conservation, encouragement to the adoption of new working practices, and the injection of a new vitality into sometimes weakened economies.

Table 7. Estimates of the benefits of rural tourism

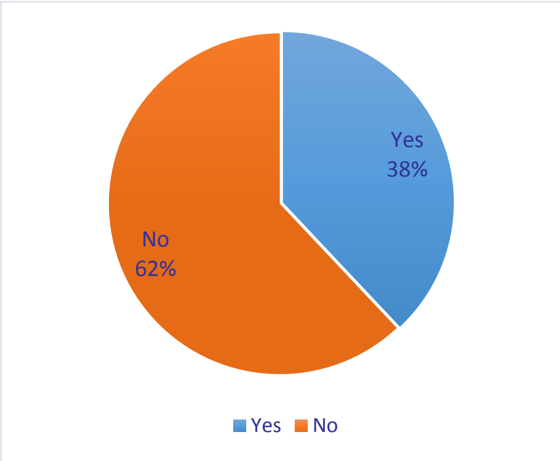
Benefits of rural tourism	Average grade	Standard deviation
To keep existing and create new jobs	3,8	1,09
To acquire additional sources of income through the sale of products (food, wood, etc.)	4,0	0,94
Opportunities for young people	3,7	1,17
Acquisition of new skills and knowledge	3,8	1,01
The sense of pride of the rural community is being revitalized	3,9	1,08
Preservation of rural culture and heritage	4,1	0,95
Sales of arts and crafts are increasing	3,6	1,14
Preservation of the natural environment	3,8	1,10
Improving life and work (asphalting of rural roads, etc.)	4,1	0,98
Preservation or restoration of the historical environment	3,6	1,31

In the area of the municipality of Prnjavor, there is a possibility of connecting agritourism with agriculture. This hypothesis was confirmed on the basis of the conducted questionnaire, i.e. the attitudes of the respondents, 97% of which believe that in the municipality of Pranjvor it is possible to connect agriculture and agritourism. Farm holders who are interested in agritourism are motivated in most cases by better sales of agricultural products and love for the countryside and agriculture, which they want to share with guests.



Graph 1.Motives for agritourism as business activity

Although farmers believe that it is possible to connect these two activities, more than half of the respondents are not interested in starting agritourism. The reason for that is ignorance and uninformedness, but also the fear of failure.

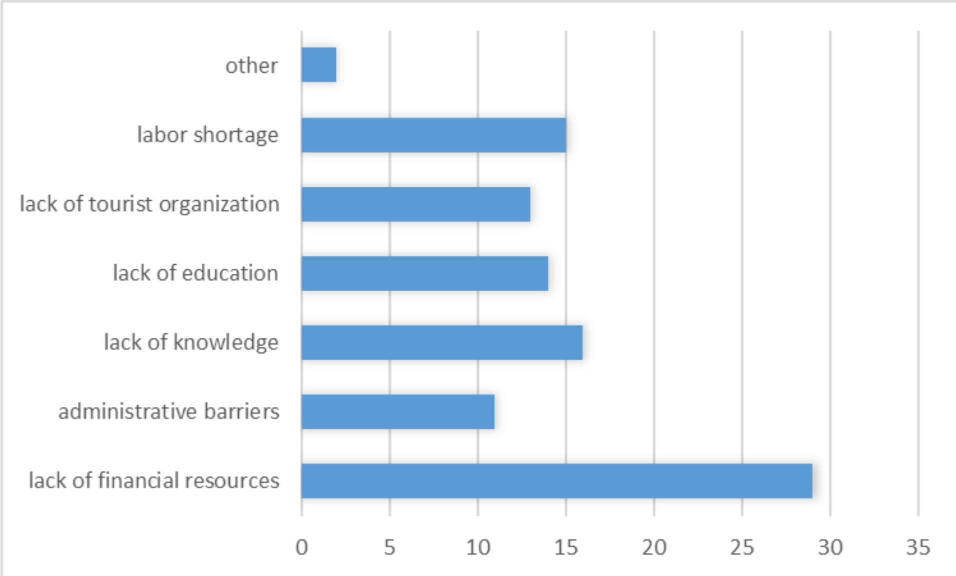


Graph 2. The interest of farm holders in agritourism

Agricultural farms in the municipality are mostly adequately equipped with infrastructure, the hosts are natives of the village, have a well-developed and diverse agricultural production, many farms are inhabited by young and educated people, these are some of the prerequisites for starting and developing agritourism and rural tourism.

Actors in the field of rural tourism in the municipality of Prnjavor are aware of the advantages and limitations for the development of rural tourism. This hypothesis was rejected based on research results. The awareness of the population about rural tourism and the opportunities it provides is not at an enviable level. Only 10% of respondents are familiar with the legislation related to rural tourism. The majority of respondents, 77% of them, have never participated in lectures/trainings on rural tourism. Respondents who participated had different opinions, more than half of them were not satisfied with the number of lectures and the information provided. When it comes to support for the development of rural tourism by the competent institutions, 43% of respondents stated that support exists and 57% that it does not exist. Respondents who stated that there is support said they were generally dissatisfied, believing that the competent institutions should be more supportive of the development of rural tourism.

Many respondents believe that the municipality of Pranjvor has nothing to offer to tourists, which means that they are not aware of the value of the potential that Prnjavor has. Although they stated that they are familiar with the concepts of agritourism and rural tourism, most respondents are not interested in engaging in this activity.



Graph 3. Barriers to engaging in rural tourism

That the awareness of rural tourism is not sufficiently developed is also shown by the fact that in the area of the municipality of Pranjvor, one rural farm is engaged in rural tourism. This farm is engaged in tourism and agriculture. They have the most guests in the summer and they are mostly children, i.e. excursion and school groups. The service provided by this farm is a picnic area, and as an additional activity on the farm they provide observation of production of crops and livestock breeding.

Other research also identify similar barriers to participate in tourism as limited knowledge about tourism, lack of capital, lack of information about tourism development, limited incentives or support from the government for tourism development (Velnisa et all, 2014, Saufi et all 2014, Tosun, 2000). According to Cigale and others, lack of experiences, knowledge, and skills negatively affect ability of farmers to adapt their offer to the demands of tourism market in Slovenia (Cigale et all, 2013).

The municipality itself, i.e. the local government, does not support rural tourism, which greatly influences this result of the research and the thinking of the population. The lack of a tourist organization in the municipality has a great impact on the level of tourism development, and thus on the attitudes and information of the local population about tourism. According to the European Commission reports, lessons learned in the period of 2007-2013 in regard to support for rural tourism record unevenly applied strategic approach(as), which are sometimes even missing (EC, 2013). According to this report national authorities opting for global approaches instead of specific local targeting. It is also noticed the tendency for rural tourism to be identified only with tourism accommodation, which affects policy orientation and narrows the investment options. Sanchez argues as urgent to incorporate participatory methodological tools designed for the rural sector in the elaboration of diagnoses for the formulation of productive projects that, through links between agriculture and tourism, strengthen countryside and thereby improve the quality of life of the rural family (Jarquin Sánchez et al.2017).

CONCLUSIONS

In the area of the municipality of Prnjavor, the most important natural resources are agricultural land and forest wealth, which represents a significant potential for the development of rural tourism. The problem is the migration of the rural-urban population and the age structure of the population in rural areas. The majority of the population are middle-aged and elderly, after which the farms remain deserted, the young population mainly moves closer to the city or to the job market.

One of the solutions to these problems can be rural tourism, or agritourism. Rural tourism would revitalize rural areas through the employment of the local population, and thus the retention of the younger population in rural areas. Rural tourism has economic and social significance, and in addition promotes environmental protection, as well as the preservation of traditions and culture characteristic of a particular rural area.

By starting rural tourism, i.e. agritourism, agricultural producers would market their products more effectively and by merging farms, the offer of agricultural products would be more diverse, which would further affect the development of agriculture and agritourism.

As mentioned earlier, the municipality of Prnjavor does not have a Tourist Organization, and the Department of Local and Economic Development and Social Activities has taken over these tasks, which is actually the biggest shortcoming when it comes to the development of any type of tourism. Through marketing activities, the tourist organization would promote the tourist potentials of the municipality of Prnjavor and in that way encourage the local population to engage in some kind of tourism. Certain lectures and trainings for the local population on rural tourism and agritourism would be of great importance, the population would be acquainted with the opportunities offered by this activity, but also with the potentials that Prnjavor has and thus would influence the awareness of the population on tourism potentials.

Rural tourism provides rural communities with the opportunity to promote and protect the environment, i.e. natural and cultural heritage. The development of rural tourism has a minimal impact on the environment and local culture, and at the same time provides an opportunity for the development of a particular area, an opportunity for additional income, new investments and the like.

The problem is in the underdeveloped awareness of the population about rural, i.e. agritourism and the opportunities it provides. By joint efforts of the competent institutions and the population, having in mind the potentials that the municipality of Prnjavor has, rural tourism could become one of the important activities in the municipality, which provides new jobs and additional income, thus affecting the development of villages and the local community.

REFERENCES

Baćac, R.,(2011): Priručnik za bavljenje seoskim turizmom, Ministarstvo turizma Republike Hrvatske;

Bojnec, Š. (2010): Rural Tourism, Rural Economy Diversification, and Sustainable Development, Academica Turistica, Year 3, No. 1–2,

Bršćić, K., Franić P., Ružić D.,(2010): Zašto agroturizam-mišljenje vlasmika, Why agrotourism-owner's opinion, Journal of Central Europen Agriculture;

Cigale, D. Lampič, B., Potočnik-Slavič, I.(2013): Interrelations between tourism offer and tourism demand in the case of farm tourism in Slovenia, European Countryside, · 4· 2013 · p. 339-355, DOI: 10.2478/euco-2013-0022

Demirović, D., Radosavac, A. (2018): Possibilities for Sustainable Rural Tourism Development in Bosnia and Herzegovina. АГРОЗНАЊЕ. 18. 237. 10.7251/AGREN1704237D.

Despotović et al (2017): Rural areas sustainability: agricultural diversification and opportunities for agri-tourism development, Agriculture & Forestry, Vol. 63 Issue 3: 47-62, 2017, Podgorica, DOI: 10.17707/AgricultForest.63.3.06

Garcia, T., Grande, I. (2005): Designing rural tourism supply in the region of Navarra, Department of Business ManagementUniversidad Pública de Navarra, ESIC MARKET

Gilbert, D.C. (1992). "Perspectivas de desarrollo de turismo rural". Revista Valenciana D'Estudis Autonomics, Vol.13, pp. 167-193.

Irshad, H. (2010): RURAL TOURISM – AN OVERVIEW, Rural Development Division, Government of Alberta, Canada

Jarquin Sánchez, N. H., Velázquez Cigarroa, E., Castellanos Suárez, J.A. (2017): Main challenges of rural development strategies through tourism and agriculture linkages in Mexico, Agriculture & Forestry, Vol. 63 Issue 4: 91-98, Podgorica, DOI: 10.17707/AgricultForest.63.4.10

Lane B., Kastenholz E., Lima J., Majewski J.,(2013): Industrial heritage and agri/rural torism in Europe;

Nacher, J. (1997): "Turismos alternativos, cooperación y desarrollo rural: un análisis crítico". Revista CIRIEC-España, Vol. 25, pp. 69-86

Nagaraju L.G.1 and B. Chandrashekara (2014). Rural Tourism and Rural Development in India. International Journal of Interdisciplinary and Multidisciplinary Studies (IJIMS), Vol 1, No.6, 42-48

Noev, N. (2013): DG AGRI/G1EAFRD support for sustainable rural tourism 2014 – 2020, "Euromeeting 2013", European Commission, Brussels (Belgium), 13/12/2013

Pesonen, J. A. (2011): Segmantation of Rural Tourists: Combining Push and Pull Motivations. Tourism and Hospitality Management. 18. 10.20867/thm.18.1.5.

Popović, G. S., Lazarević, S. (2021): Skadar Lake cultural landscape and architectural heritage-Potential for the Development of Fisheries and Rural Tourism. Agriculture and Forestry, 67(1): 255-269. DOI: 10.17707/AgricultForest.67.1.21

Saufi, A., O'Brien, D., Wilkins, H. (2014): Inhibitors to host community participation in sustainable tourism development in developing countries, Journal of Sustainable Tourism, 22:5, 801-820, DOI: 10.1080/09669582.2013.861468

Šehić-Kršlak S.,(2018): Koncept ruralnoga turizma u funkciji privrednoga razvoja Bosne i Hercegovine, Fakultet za menadžment i poslovnu ekonomiju, Univerzitet u Travniku;

Sharpley, R. (2000): Tourism and Sustainable Development: Exploring the Theoretical Divide, Journal of Sustainable Tourism, 8:1, 1-19, DOI: 10.1080/09669580008667346

Standing working group for regional rural development (SWG), (2020): ECONOMIC diversification policies and rural tourism in South East Europe/[edited by Suzana ĐorđevićMilošević et al.]. - Skopje

Streifeneder, T.,Dax T. (2020): Agritourism in Europe: Enabling Factors and Current Developments of Sustainable On-Farm Tourism in Rural Areas, Source Title: Global Opportunities and Challenges for Rural and Mountain Tourism, DOI: 10.4018/978-1-7998-1302-6.ch003 (pages 40-58)

Tosun, C. (2000): Limits to community participation in the tourism development process in developing countries, Tourism Management Volume 21, Issue 6, December 2000, Pages 613-633

Velnisa, F., Modilih, S., Mogindol, S., Johnny, C., Thamburaj, A. (2014): Community Participation and Barriers in Rural Tourism: A Case Study in Kiulu, Sabah. SHS Web of Conferences. 12. 10.1051/shsconf/20141201003.

Fathalipour, B., B., Ahadnezhad R., A., Abbasi, A. (2022): Changes in antioxidant enzymes of Hordeum vulgare L. using the endophytic fungus Piriformospora indica and the bacterium Azospirillum spp. under drought stress. Agriculture and Forestry, 68 (1): 261-283. doi:10.17707/AgricultForest.68.1.17

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CHANGES IN ANTIOXIDANT ENZYMES OF BARLEY (*Hordeum vulgare* L.) USING THE ENDOPHYTIC FUNGUS *PIRIFORMOSPORA INDICA* AND THE BACTERIUM *AZOSPIRILLUM* SPP. UNDER DROUGHT STRESS

SUMMARY

Piriformospora indica and *Azospirillum* spp. due to great ability to effectively improve plant growth and stress tolerance, have been considered significantly in recent decades. The present study was conducted as factorial based on a randomized complete block with three replications in the research field of Plant Production Engineering and Genetics, Faculty of Agriculture, Maragheh University. The experimental treatments included three levels of stress (full irrigation, irrigation at 70% of field capacity, and irrigation at 50% of field capacity), endophytic fungus *Piriformospora indica* (non-use and use) and bacterium *Azospirillum* (non-use and use). The results of the present study showed that the interaction between drought stress and using fungi and bacteria had a significant effect at the probability level of 1% on leaf area, chlorophyll a, the activity of glutathione reductase, ascorbate peroxidase, and catalase; and the content of malondialdehyde, hydrogen peroxide, superoxide dismutase, Fe-SOD isozymes, Mn-SOD, and proline at the probability level of 5% on Cu/Zn-SOD isozyme. The simple effects of the studied treatments were also significant on the parameters of chlorophyll b and carotenoids at the statistical level of 1%. The highest content of superoxide dismutase, Cu/Zn-SOD, Fe-SOD, Mn-SOD isozymes, ascorbate peroxidase, catalase peroxidase and glutathione reductase was obtained from inoculation with bacteria and fungi in irrigation treatment at 50% of field capacity resulting in a reduction by 47.01 and 33.93% in the content of hydrogen peroxide and malondialdehyde in irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria compared to the non-use of fungi and bacteria in the same treatment. So, it can be concluded that the combined use of *Piriformospora indica* and *Azospirillum* spp. under conditions of severe drought stress can play an important role in modulating Barley growth.

Keywords: Ascorbate peroxidase, catalase, hydrogen peroxide, lipid peroxidation, yield

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the main sources of animal nutrition. The plant is the fourth largest crop in the world after wheat, rice and maize (Biel and Jacyno, 2013). According to the World Food Organization (FAO), in 2019 the global cultivation area of Barley is 47 million hectares and the global mean production of this plant is 31,000 kg per hectare.

For the importance of water, it should be mentioned that out of 70% of the water that covers the surface of the earth, only 2.5% is sweet and can be involved in meeting the needs of plants and organisms (Gleick and Palaniappan, 2010). Drought stress is one of the most important environmental stresses that affects more than 45% of agricultural lands, about 38% of the world's population (Ashraf and Foolad, 2007). By 2030, nearly 2.8 billion people in 48 countries will face drought stress, according to the Food and Agriculture Organization of the United Nations (Fao, 2009). During drought stress, plants show different physiological and biochemical changes, the most important of which is the reduction in the production of pure photoassimilates due to the closure of the stomata (Ashraf and haris, 2004). Under such conditions, the reduction in carbon dioxide gas stabilization causes a severe reduction in the photosynthetic electron transfer chain, electron leakage to the oxygen molecule and the formation of reactive oxygen species (Anjum et al., 2003). Different types of reactive oxygen species include superoxide radical ($O2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO2^{\cdot}$), hydrogen peroxide (H2O2), radical alcohol ($RO^{\cdot}$), radicalproxy ($ROO^{\cdot}$), single oxygen (1O2) and carbonyl excited (RO*) (Dismukes et al., 2001; Karuppanapandian et al., 2006a, 2006 b, 2006 c, 2008, 2011; Karuppan- apandian and Manoharan, 2008; Vellosillo *et al*., 2010). Under drought stress, increasing the production of reactive oxygen species causes oxidative degradation of lipids (Suzuki and Mittler, 2006), proteins (Waszczak *et al*., 2018) and nucleic acids (Karuppanapandian *et al*., 2011), which with the continuation of stress leads to planned death in the cell (PCD, Vranova *et al*., 2002; Manoharan *et al*., 2005; Karuppanapandian *et al*., 2011). The mechanisms for coping with drought stress in plants include a combination of stress prevention, tolerance, recovery and avoidance strategies (Malinowski and Belesky, 2000).

However, in crops, the main role is played by reducing the destructive effects of drought stress due to the presence of antioxidants and antioxidant enzymes (Cossania *et al*., 2012). Enzymes are essentially catalysts for physiological reactions in plants and are the most sensitive agents of physiological changes in plants under environmental stress (Foyer and Shigeoka, 2011). The antioxidant system formed in higher plants against these threats consists of several special enzymes, including glutathione peroxidase, catalase, superoxide dismutase, and ascorbate peroxidase (Lisar *et al*., 2012). This defense system is able to collect reactive oxygen radicals and minimize their adverse effects (Tan *et al*., 2006) that finally leads to plant tolerance to environmental stresses, including drought stress (Sharma *et al*., 2012).

Using biological methods based on potential of beneficial soil microorganisms for establishing symbiosis relationships with plants has been mentioned as an effective solution to increase the tolerance of crops to environmental stresses (Bacilio *et al*., 2004). Endomycorrhizal fungus *Piriformospora indica* belongs to the group of mycorrhizal fungi, the order Sebacinales, Hymenomycetes and the division basidiomycota (Kumar *et al*., 2011). Many researchers have reported a correlation between *P. indica* and the roots of different plant species and confirmed their positive effect on the yield of these plants (Dolatabadi *et al*., 2011; Oelmuller *et al*., 2009; Varma *et al*., 2012). Hosseini et al., (2017) reported that drought stress affected fresh weight of roots and stems, root volume, leaf area, relative water content, leaf water potential, and chlorophyll content severely. The presence of endophytic fungi can reduce the adverse effects of environmental stresses. In this regard, Yaghoubian et al., (2014) showed that drought stress caused increasing hydrogen peroxide content and lipid peroxidation in wheat, but inoculation of these plants with *G. mosseae* and *P. indica* increased the activity of antioxidant enzymes catalase, ascorbate peroxidase and peroxidase and finally reduced reactive oxygen species and plant growth.

Plant growth-promoting bacteria are beneficial bacteria that promote the rooting of crops and increase their growth and yield. One of the most important bacteria is *Azospirillum* spp., which in addition to stabilizing atmospheric nitrogen (Döbereiner and Day, 1976), also can mineralize nutrients from the soil (Bashan et al. 2004; Fibach-Paldi et al., 2012). In addition, several researchers report that *Azospirillum* spp.can play a positive role in reducing the negative impacts of abiotic stresses (Casanovas *et al*., 2003; Creus *et al*., 2004; Barassi *et al*., 2006; Pereyra *et al*., 2006; Creus *et al*., 2010). The most important effects of *Azospirillum* spp. under drought stress can be altered levels of some plant hormones such as abscisic acid, indoleacetic acid (Dobbelaere *et al*., 2003; Spaepen *et al*., 2007), direct absorption of water by hyphae and transfer to the host, increased leaf gas exchange, increased activity of antioxidant enzymes, assimilation of nitrate and phosphorus, increased hydraulic conductivity of leaf water, osmotic regulation and increased flexibility of cell membranes (Bashan and de-Bashan, 2010).

Drought stress in fields of Barley affects the quantitative and qualitative yield of this crop. It is obvious that reducing the effects of drought stress can play an effective role in improving the current situation. In the present study, it was attempted to investigate the effect of using the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. in order to increase the moderation of Barley and changes in the defense systems of this plant under different moisture conditions.

MATERIAL AND METHODS

The present study was conducted in the research field of the Faculty of Agriculture of Maragheh University with geographical coordiantes of latitude 37.37 ´ N, longitude 46/27 ´E and altitude 1552 m above sea level. The climate of Maragheh is temperate, cold and relatively humid. Also, the annual rainfall in this city is about 330 mm and its icy days are about 114 days per year. The present study was conducted as factorial based on a randomized complete block design with three replications. The studied factors are three levels of stress (complete irrigation, irrigation at 70% of field capacity, and irrigation at 50% of field capacity), endophytic fungus *Piriformospora indica* (non-use and use) and *Azospirillum* bacteria (non-use and use). Before performing the test, a sample of field soil was prepared from a depth of 0 to 30 cm and after mixing, a composite soil sample was transferred to the laboratory to determine some physical and chemical properties, the results of which are shown in the Table 1.

Table 1. Results of physical and chemical decomposition of soil used before planting

Properties measured in the tested soil	Amounts	Properties measured in the tested soil	Amounts
Soil texture	Lumi - Sandy	Total nitrogen (percentage)	0.06
The acidity of saturated mud	7.51	Absorbable phosphorus (mg / kg - Olson method)	3.81
Electrical conductivity (dS / m)	0.46	Absorbable potassium (mg / kg - ammonium acetate)	346
Equivalent calcium carbonate (percentage)	9.61	Absorbable zinc (mg / kg-DTPA)	0.38
Organic carbon (percentage)	0.32	Absorbable manganese (mg / kg-DTPA)	1.56
Saturation humidity (percentage)	46	Absorbable iron (mg / kg-DTPA)	3.26

In order to apply drought stress, all the treated treatments up to 4-6 leaf stage were fully irrigated. Then, irrigation times of the field were determined by measuring the soil moisture by weight method through soil sampling in the middle of each day from the depth of root development in different treatments and reaching the desired moisture (Martin *et al*., 1990). In order to apply drought stress, the amount of irrigation water for each plot was calculated taking into account the depth of root development (50 cm), plot area and soil moisture capacity in cubic meters and added to each plot in a certain amount (Rostamza *et al.*, 2011).

$$In = \frac{(Fci - \theta i) \times D \times A}{100}$$

In: Volume of water use, Fci: Soil moisture at field capacity, ϴi: Soil moisture content during sampling, D: Appropriate depth of root penetration, A: Plot area used.

In order to extract superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX), 0.5 g of each leaf sample was weighed with a 000 scale and homogenized using cold porcelain mortar and liquid nitrogen. The prepared homogenate was centrifuged at 15000 rpm at 4 °C for 15 min after transfer to the microtube (Sairam *et al*., 1998).

Sairam *et al*., (2002) method was used to measure the activity of superoxide dismutase and its isozymes. According to this method, the reaction complex consisted of sodium carbonate, potassium phosphate buffer, distilled water, EDTA, methionine and extractive enzyme. By adding riboflavin, the reaction started and after stopping the reaction, the absorbance of the samples was read at the wavelength of 560 nm using a spectrophotometer (Sairam *et al*., 1998).

Catalase activity was measured according to Aebi (1984) method. The reaction complex consists of potassium phosphate buffer, hydrogen peroxide, double distilled water and enzymatic solution. The adsorption of the reaction complex was read at the wavelength of 290 nm and the enzyme activity was calculated using extinction coefficient of 36.16 $mmol^{-1}$ cm^{-1}. In order to measure the activity of ascorbate peroxidase, a reaction complex including phosphate buffer, double distilled water, hydrogen peroxide, EDTA, ascorbate and enzymatic solution extracted. The adsorption of the reaction complex was read at the wavelength of 290 nm using a spectrophotometer and the enzyme activity was calculated using extinction coefficient of 2.8 $mmol^{-1}$ cm^{-1} (Sairam *et al*., 1998).

In order to measure the activity of guaiacol peroxidase, the reaction complex consists of phosphate buffer, guaiacol, hydrogen peroxide, EDTA and the extracted enzyme solution. The increase in absorption was recorded using a spectrophotometer for 60 seconds at a wavelength of 470 nm and obtained using extinction coefficient of 26.6 $mmol^{-1}$ cm^{-1} (Yoshimura *et al*., 2000).

Malondialdehyde was measured by Stewart and Bewley (1980) method. Homogenized leaf samples were centrifuged at 15000 × g for 10 min. The resulting solution was mixed with trichloroacetic acid and thiobarbituric acid and the resulting complex was centrifuged again at 10000 × g for 10 min after transfer to a cold-water bath. The absorbance of the samples was recorded at wavelengths of 532 and 600 nm using a spectrophotometer. The content of malondialdehyde was obtained from the difference between the absorption waves and extinction coefficient 155 $mmol^{-1}$ cm^{-1}.

In order to estimate the content of hydrogen peroxide, the digested samples were centrifuged with trichloroacetic acid at 12000 × g for 15 min. The reaction complex containing supernatant, phosphate and potassium iodide was prepared and their adsorption was read at the wavelength of 390 nm using a spectrophotometer (Chen *et al*., 2000).

Before statistical analysis of data and analysis of variance, normal data distribution test and errors were performed. The mean comparison was performed by Duncan's multiple range at a probability level of 1%. GenStat 12, Excel and SPSS17 software were used for data analysis and diagram drawing.

RESULTS AND DISCUSSION

According to the analysis of variance, the interaction between drought stress and the use of fungi and bacteria had a significant effect on the probability level of 1% on leaf area, chlorophyll a, the activity of glutathione reductase, ascorbate peroxidase, and catalase, and the content of malondialdehyde, hydrogen peroxide, superoxide dismutase, Fe-SOD isozymes, Mn-SOD, and proline at the statistical level of 5% on Cu/Zn-SOD isozyme (Table 2). The simple effects of each treatment were significant on chlorophyll b and carotenoids at the probability level of 1% (Table 2).

Table 2. Analysis of variance of parameters measured using the endophyte fungus *Piriformospora indica* and *Azospirillum* spp. in different humidity conditions

Sources of changes	Degrees of freedom	average of squares						
		Lef area	Chlorophyl l a	Chlorophyl l b	Cartonoid	Superoxide dismutase	Cu/Zn-SOD	Mn-SOD
Repetition	2	2.389	2.389	98.53	15.5	3.3190	0.07381	0.3701
Drought stress	2	824.541**	824.541**	7591.69**	15792.1**	329.1600**	7.95363**	11.7005**
inoculated	3	137.116**	137.116**	5171.70**	18954.5**	53.1575**	1.06360**	2.6460**
Drought stress * Inoculation	6	34.768**	34.768**	60.06ns	288.4ns	12.1503**	20.237*	1.2533**
Error	22	1.633	1.633	29.07	201.1	0.4257	0.07446	0.1547
Coefficient of variation		3	3	5.9	8.3	5.7	18.3	12.3

Sources of changes	Degrees of freedom	average of squares						
		Fe-SOD	Catalase	Ascorbate Peroxidase	glutathione peroxidase	hydrogen peroxide	Malondialdehyde	Proline
Repetition	2	2.7191	0.12564	0.00259	0.03301	0.25617	25.514	7.926
Drought stress	2	2.45184**	2.58139**	2.45184**	8.90215**	11.90521**	802.273**	235.648**
inoculated	3	22.4641**	0.46357**	2.30755**	1.82285**	3.60833**	168.101**	110.025**
Drought stress * Inoculation	6	5.1014**	0.15018**	0.62351**	0.50145**	0.69514**	35.404**	31.494**
Error	22	0.2010	0.01460	0.03444	0.03181	0.05752	2.689	2.919
Coefficient of variation		6.6	20.5	14.3	9.9	9.8	5.2	5.7

ns, * and ** are non-significant, significant at the 5% and 1% probability levels, respectively.

The results of analysis of variance of the present study showed that the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress had a significant effect on Hordeum vulgare L. leaf area (Table 2). According to the mean comparison (Table 3), the highest and lowest leaf area were obtained from irrigation treatment at 90% of field capacity and combined use of fungi and bacteria and irrigation treatment at 50% of field capacity and no inoculation, respectively. Using fungi and bacteria at 50, 75 and 90% of field capacity showed 12.72, 14.49, 40.75% increase compared to non-use of fungi and bacteria, respectively (Figure 1). Kim et al., (2017) also reported that leaf area index is the most morphologically sensitive trait to drought stress that has a high correlation with biological and economic yield. Drought stress has the greatest effect on the optical part of photosynthesis. Liu and Stützel (2004) found that drought stress significantly reduced dry weight and leaf area in four genotypes of Amaranthus spp. In contrast, the colonization of maize roots by *P. indica* under drought stress increased maize leaf area (Xu et al., 2017).

Table 3. Mean comparisons of traits of antioxidant enzymes and malondialdehyde under the influence of endophytic fungus *Piriformospora indica* and *Azospirillum* spp. under drought stress conditions

Drought stress	**Treatment**	**Superoxide dismutase**	**Cu/Zn-SOD**	**Mn-SOD**	**Fe-SOD**	**Glutathione peroxidase**	**Proline**
Irrigation at 90% of field capacity	Lack of insemination	5.74h	0.623f	2.170c	2.943e	0.937g	24.94d
	Inoculation with bacteria	5.78h	0.623f	2.233c	2.927e	0.847g	24.69d
	Inoculation with mushrooms	6.00h	0.700ef	2.337c	2.960e	0.850g	25.86d
	Inoculation with fungi and bacteria	6.39h	0.723ef	2.473c	3.193e	0.883g	24.47d
Irrigation at 75% of field capacity	Lack of insemination	9.80g	1.113def	2.767bc	5.897d	1.123fg	36.33ab
	Inoculation with bacteria	9.91fg	1.230cdef	2.987bc	5.690d	1.687ef	31.88bc
	Inoculation with mushrooms	12.33de	1.500bcde	3.047bc	7.873c	2.103de	29.88cd
	Inoculation with fungi and bacteria	15.99bc	2.223ab	3.333bc	10.433b	2.787bc	25.19d
Irrigation at 50% of field capacity	Lack of insemination	11.97ef	1.860bcd	2.678bc	7.420c	1.853de	40.93a
	Inoculation with bacteria	14.29cd	2.077bc	3.757b	8.457c	2.260cd	36.20ab
	Inoculation with mushrooms	17.93b	2.247ab	5.103a	10.583b	2.857ab	29.68cd
	Inoculation with fungi and bacteria	21.45a	2.997a	5.483a	12.967a	3.380a	27.94cd

According to the results of the present study, drought stress caused a reduction in leaf area and the combined use of fungi and bacteria improved the leaf area compared to the non-use of fungi and bacteria under stress and even desired moisture conditions. According to the analysis of variance, using fungi and bacteria under different moisture conditions had a significant effect at the probability level of 1% on the content of chlorophyll a (Table 2). As shown in Figure 2, the highest content of chlorophyll a was recorded in irrigation treatment at 90% of field capacity and combined use of fungi and bacteria, respectively. Inoculation with fungi and bacteria at 50, 75 and 90% of field capacity showed an increase in chlorophyll a of Hordeum vulgare L. by 12.72, 14.49 and 40.75%, respectively, compared to the control. In this regard, Ommen et al., (1999) in spring wheat, Manivannan et al., (2007) in sunflower, and li et al., (2006) in barley reported a reduction in chlorophyll a content due to drought stress. Yaghoubian et al., (2014) showed that inoculation with *G. mosseae* and *P. indica* increased chlorophyll a content of wheat under drought stress. Zarea et al., (2012) also reported that using *Piriformospora indica* and *Azospirillum* increased chlorophyll a content in wheat under salinity stress. The reason for this in subsequent reports was the positive relationship between phosphorus concentration and chlorophyll content in plants. On the one hand, the reason for the reduction in chlorophyll content under drought stress can be the degradation of chloroplast thylakoid membranes and chlorophyll optical oxidation due to increased activity of reactive oxygen species and high chlorophylase activity (Ashraf, 2009). Drought and salinity stresses increase the concentration of growth regulators such as abscisic acid and ethylene, which stimulate chlorophylase and cause the decomposition of chlorophyll (Orabi et al., 2010). The combined use of fungi and bacteria in Hordeum vulgare L. under drought stress improved water uptake of the plant compared to the non-use of fungi and bacteria under moisture stress, which in turn reduced the activity of reactive oxygen species and chlorophylase and prevent degradation of chloroplast thylakoid membranes and chlorophyll optical oxidation, which can also result in improved plant growth.

According to the analysis of variance, the simple effects of using fungi and bacteria and application of drought stress were significant at the statistical level of 1% on the content of chlorophyll b (Table 2). According to the mean comparison, the highest value of this parameter was recorded in irrigation treatment at 90% of field capacity (113.42 mmol / g fresh weight) and combined use of fungi and bacteria (122.11 mmol / g fresh weight) (Table 4). Many researchers have reported an increase in chlorophyll b content using fungi and bacteria in various plants (Yaghoubian et al., 2014; Hoseini et al., 2017). several researchers have reported a reduction in chlorophyll b content under drought stress in different plants (Ommen et al., 1999; Manivannan et al., 2007; Gregersen and holm, 2007; li et al., 2006). Drought stress causes oxidative stress, which in turn causes the production of active oxygen in chloroplasts, which are very harmful radicals and have adverse effects on the photosystem (Cruz de Carvalho, 2008). And by increasing amount and time the degradation process of chlorophyll pigments increases. Chlorophyll content in crops is one of the important factors in maintaining photosynthetic capacity. Agami et al., (2017) reported that chlorophyll content in wheat inoculated with *Azospirillum* spp. increased under stress. Barassi et al., (2000) reported that *Azospirillum* spp. is a growth-promoting bacterium under drought and salinity stress. Many reports have proven the ability of this bacterium to resolve drought stress (Casanovas et al., 2003). Symbiosis with mycorrhizal fungi can increase the biosynthesis of chlorophyll in crops by improving the absorption of magnesium and phosphorus (kadian et al., 2013). In this regard, researchers have found an increase in chlorophyll a and b in plants inoculated with *P. indica* due to improved plant water status and absorption of minerals such as magnesium (Giri and Mukerji, 2004). Harman et al., (2021) stated that endophytic fungus *P. indica* played an effective role in maintaining and stabilizing photosynthesis by having a positive effect on proteins involved in the process of photosynthesis and Calvin cycle and increasing their expression. The degradation of and reduction in chlorophyll under drought stress can be compatible because by reducing chlorophyll, the electron excited during photosynthesis reduces and consequently the damage caused by the formation of oxygen free radicals is reduced (Kranner et al., 2002). Limited CO2 stabilization due to stress leads to a reduction in carbon by Calvin cycle and $NADP^+$ oxide as electron acceptor in photosynthesis. By reducing Ferredoxin (Fd) during electron transfer, the electron maybe transferred from PSI to O2 to form $O2^{\cdot -}$ by a process called the Mahler reaction. The environmental stress disturbs the balance between light absorption and energy use and increases reactive oxygen.

Table 4. Comparison of the mean simple effects of drought stress and lack of inoculation and inoculation with fungi and bacteria on chlorophyll b and carotenoids

Treatment	Chlorophyll b	carotenoid
Lack of insemination	65.67d	123.00d
Inoculation with bacteria	81.00c	149.60c
Inoculation with mushrooms	94.78b	182.80b
Inoculation with fungi and bacteria	122.11a	229.40a
Drought stress	Chlorophyll b	carotenoid
Irrigation at 90% of field capacity	113.42a	208.20a
Irrigation at 75% of field capacity	95.50b	169.60b
Irrigation at 50% of field capacity	63.75c	135.80c

According to the analysis of variance, the simple effects of using fungi and bacteria and application of drought stress were significant at the statistical level of 1% on the content of carotenoids (Table 2). According to the mean comparison, the highest value of this parameter was recorded in irrigation treatment at 90% of field capacity (208.2 mmol / g fresh weight) and the treatment of the combined use of fungi and bacteria (229.4 mmol / g fresh weight) (Table 4). As a non-enzymatic antioxidant, carotenoids stop the oxidation process through neutralizing free radicals and reduce the effects of stress. They are part of tetraterpene compounds and are considered as protectors for chlorophylls by converting single oxygen to ternary oxygen and reducing the damage caused by the presence of reactive oxygen species (Zur et al., 2000). Carotenoids protect the plant against oxidative stress by removing all types of active oxygen (Mahajan and Tuteja, 2005). Carotenoids accumulate harmful oxygen species and protect chlorophyll through interfering with the xanthophyll cycle. In this regard, Abid et al., (2018) stated that drought stress caused a reduction in carotenoid concentration in wheat. On the one hand, inoculation with *P. indica* and *Azospirillium* in several reports reduced the effects of drought stress and moderated carotenoid levels (Basak et al., 2011).

According to the analysis of variance, using fungi and bacteria under different moisture conditions had a significant effect on the activity of superoxide dismutase at the probability level of 1% (Table 2). As shown in Table 3, the highest superoxide dismutase activity was recorded in irrigation treatment at 90% of field capacity and the combined use of fungi and bacteria. Inoculation with fungi and bacteria in irrigation treatments at 50 and 75% of field capacity showed an increase by 79.19 and 63.19%, respectively, compared to the control (Table 3). Superoxide dismutase combines two superoxide radical molecules with hydrogen and finally produces two molecules of water and oxygen, hence, reducing the adverse effects of superoxide radicals on plant cells (Wang *et al*., 2018). In a study by Chakraborty *et al*., (2013) a rapid reduction in the activity of superoxide dismutase was recorded after 3 days of applying drought stress, while using bacteria, the activity of this enzyme reduced much less rapidly. The activity of superoxide dismutase increased in plants inoculated with different fungi under various environmental stresses including drought, salinity and heavy metals (Gururani *et al*., 2013). According to analysis of variance, using the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under different moisture conditions had a significant effect on the activity of Fe-SOD and Mn-SOD isozymes at the probability level of 1% and Cu/Zn-SOD isozyme at the statistical level of 5% (Table 2). The highest and lowest Fe-SOD isozyme activity was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (12.967 enzymatic units per mg of protein) and irrigation treatment at 90% of field capacity and using fungi, respectively (2.927 enzyme units per mg of protein) (Table 3). The highest and lowest Cu/Zn-SOD isozyme activity was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (2.997 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity and using fungi (0.623 enzyme units per mg of protein), respectively (Table 3). The highest and lowest Mn-SOD isozyme activity was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (5.483 enzymatic units per mg of protein) and irrigation treatment at 90% of field capacity and non-use of fungi and bacteria (2.170 enzyme units per mg of protein), respectively (Table 3). Simultaneously with the expression of Cu/Zn-SOD and ascorbate peroxidase genes in chloroplasts of transgenic plants, tolerance to abiotic stresses was observed (Lee et al., 2007). Superoxide dismutase is one of the key enzymes in the immune system of plant cells, which plays an important role in the oxidation of cellular biological matter, which converts superoxide radical into hydrogen peroxide (Alscher *et al*., 2002). Reducing superoxide dismutase leads to superoxide radical increase, which in turn causes damage to plant cells, followed by metabolic disorders and finally programmed cell death (Breusegem *et al*., 2001). According to some reports, the activity of superoxide dismutase increases at the beginning of drought stress (Baisak *et al*., 1994), but by increasing drought stress, the activity of this enzyme also reduces. The combined use of fungi and bacteria, in other words, in addition to increasing the activity, increases the period of activity of this enzyme under stress. In the present study, it was found that the combined use of fungi and bacteria simultaneously by increasing levels of drought stress increases the activity of this enzyme to respond to the occurrence of stress. However, a reduction in the activity of superoxide dismutase under stress has also been recorded by Yong *et al*., (2006).

According to the analysis of variance, using fungi and bacteria under drought stress had a significant effect on the activity of ascorbate peroxidase at the probability level of 1% (Table 2). According to the mean comparison, inoculation with fungi and bacteria at 50 and 75% of field capacity showed an increase by 219.73 and 76.92%, respectively, compared to non-inoculation in the activity of this enzyme (Figure 3). The highest and lowest activity of ascorbate peroxidase was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (2.900 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity and no inoculation (0.787 enzyme unit per mg of protein), respectively (Figure 3). Ascorbate peroxidase, by combining hydrogen peroxide and ascorbic acid and producing water and dihydroascorbate in chloroplasts and cytosols of crops, causes the accumulation of hydrogen peroxide, especially under environmental stress (Asada, 2000). Reduced ascorbate peroxidase activity in wheat was reported by Sofo *et al*., (2015) The researchers stated that reducing this enzyme increased hydrogen peroxide. When the amount of hydrogen peroxide increases, the activity of some enzymes stops in Calvin cycle, such as ribulose-5-phosphate kinase and bisphosphatases and isozymes of superoxide dismutase. Heidari *et al.*, (2009) reported an increase in ascorbate peroxidase activity in sorghum under moderate stress, which was introduced as the main factor of plant tolerance to stress and reduction in its adverse effects. Further studies have shown that plant type and level of stress have a significant effect on the process of enzyme changes. Xu *et al*., (2018) stated that *P. indica* can increase the synthesis of auxin by coexisting with plant roots. Sarker *et al*., (2018) stated that wheat inoculated with this fungus modulated the enzymatic activity of ascorbate peroxidase due to increased water absorption and reduced effects of drought stress. Caverzan *et al*., (2012) showed that the presence of this fungus increased the expression of ascorbate peroxidase gene under salinity and drought stress. Vurukonda *et al*., (2016) reported that growth-promoting bacteria (PGPR) accumulated hydrogen peroxide and increased plant tolerance through increasing mRNA expression of ascorbate peroxidase, superoxide dismutase and catalase in potato.

According to analysis of variance, the interaction between the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under different moisture conditions had a significant effect on the level of catalase activity at the probability level of 1% (Table 2). According to the mean comparison, the highest and lowest activity of this enzyme was obtained from irrigation treatments at 50% of field capacity and the combined use of fungi and bacteria (1.4080 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity, and using fungi (0.0520 units of enzyme per mg of protein), respectively (Figure 4). The combined use of fungi and bacteria at 50 and 75% of field capacity compared to non-inoculation treatment caused an increase by 219.73 and 197.15% in this enzyme, respectively (Figure 4). Catalase reduces the effects of oxidative stress on crops by removing hydrogen peroxide produced in peroxisome by oxidases involved in β fatty acid oxidation, cell respiration, purine catabolism (Mittler, 2002; Vellosillo *et al*., 2010). With the activity of this enzyme, hydrogen peroxide is converted into water and oxygen and the adverse effect of this harmful matter is reduced. Yamazaki *et al*., (2003) considered the high level of hydrogen peroxide as a direct factor in preventing carbon dioxide stabilization because some of the enzymes of Calvin cycle are highly sensitive to hydrogen peroxide. Shao et al., (2005) Heidari *et al*., (2009) and Srivall *et al*., (2004) considered the increase in catalase activity as effective on stress tolerance. Yaghoubian *et al*., (2014) found that using *G. mosseae* and *P. indica* increased the activity of wheat catalase and ascorbate peroxidase under drought stress. In general, bacterial residues depend on their contribution to reducing abiotic stress and plant growth (Dimkpa et al., 2009).

According to the analysis of variance, the interaction between fungi and bacteria and drought stress had a significant effect on the activity of glutathione Peroxidase at the probability level of 1% (Table 2). According to the mean comparison, the highest and lowest activity of this enzyme was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (3.380 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity and using fungi (0.847 enzyme units per mg of protein), respectively (Table 3). The combined use of fungi and bacteria at 50 and 75% of field capacity showed an increase by 82.40, 148.17% compared to non-use of fungi and bacteria, respectively, and irrigation treatment at 90% of field capacity with the combined use of fungi and bacteria reduced glutathione Peroxidase activity by 5.76% (Table 3). Gupta et al., (2021) showed that the activity of glutathione Peroxidase in roots and stems of rice inoculated with P. indica increased under salinity stress. Glutathione Peroxidase catalyzes the combination of oxidized glutathione with adenine nicotine dinucleotide phosphate, leading to the formation of glutathione. Glutathione is involved in the regeneration of ascorbate from dehydroascorbate by dehydroascorbate reductase. Therefore, it can be concluded that inoculation with fungi and bacteria through increasing the activity of glutathione reductase under drought stress causes glutathione accumulation and reduction in ascorbic acid in ascorbic acid-glutathione cycle, and adverse effects of superoxide radical, hydroxyl radical and hydrogen peroxide on plant cells.

The results of analysis of variance of the present study showed that using the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress had a significant effect on the amount of hydrogen peroxide of Barley at the probability level of 1% (Table 2). According to the mean comparison, the highest and lowest accumulations of hydrogen peroxide (4.56 and 1.09 mmol / g, respectively) was obtained from irrigation treatment at 50% of field capacity and no inoculation of fungi and bacteria and irrigation treatment at 90% of field capacity and the combined use of fungi and bacteria, respectively (Figure 5). The amount of this matter in the treatment of the combined use of fungi and bacteria in irrigation treatment at 50% of field capacity was recorded about 2.42 mmol / g fresh weight (Figure 5). In other words, only with the combined use of fungi and bacteria in irrigation treatment at 50% of field capacity, the amount of hydrogen peroxide accumulation reduced by 46.92% (Figure 5). Hydrogen peroxide (H_2O_2) is one of the most toxic forms of depleted oxygen reduction, which has detrimental effects on plant cell metabolism, including the oxidation of thiol groups. Also, the activity of some Calvin cycle enzymes such as ribulose 5-phosphate kinase and bisphosphatases is reduced or stopped with high amount of hydrogen peroxide. As a result, due to the reduction in $NADP^+$ / NADPH, H^+ ratio, the production of reactive oxygen species also increases. In addition, Mn-SOD and Cu/Zn-SOD isozymes are sensitive to high amount of this matter and lose their activity. Costa et al., (2005) stated that accumulation of hydrogen peroxide in addition to damaging membranes causes damage to macromolecules such as DNA and proteins. According to reports by Sairam et al., (2002) drought and salinity stress cause a significant increase in the amount of hydrogen peroxide inside the cell. Sairam et al., (2002) stated that the accumulation of hydrogen peroxide increased the lipid peroxidation and reduced the membrane stability index in wheat leaf cells. Under normal conditions, plant cells continuously produce some hydrogen peroxide, which is decomposed by enzymatic antioxidants such as catalase and ascorbate peroxidase. Under drought stress, the production of hydrogen peroxide increases, plant cells are synthesized and activity of these enzymes increases to some extent to minimize the negative impacts of the production of this matter. In the present study, the activity of two enzymes ascorbate peroxidase and catalase under drought stress was higher than the control. Different species and strains of Azospirillium bacteria directly affect the activity of these enzymes by modulating the intensity of stress. These bacteria inhibit the production of ethylene by producing aminocyclopropane and carboxylic acid deaminase, and finally reduce the level of antioxidant enzymes. Reduced hydrogen peroxide accumulation, growth modulation of different plants and the activity of catalase and ascorbate peroxidase have also been reported by Sofa et al., 2015 Studies by Baltruschat et al., (2008) showed that P. indica stimulates the accumulation of ascorbate in the root cells of host plants. Ascobic acid acts as a raw material in glutathione ascorbate cycle to detoxify hydrogen peroxide and regulate the production of other types of reactive oxygen species.

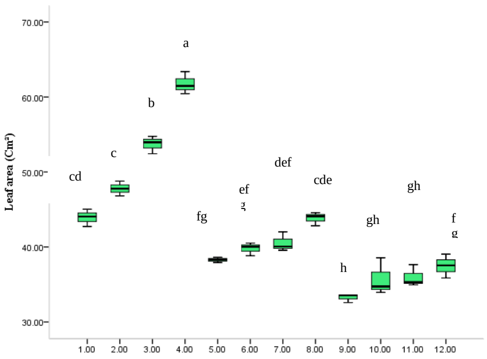

Figure 1. Changes in leaf area with inoculation of barley under drought stress

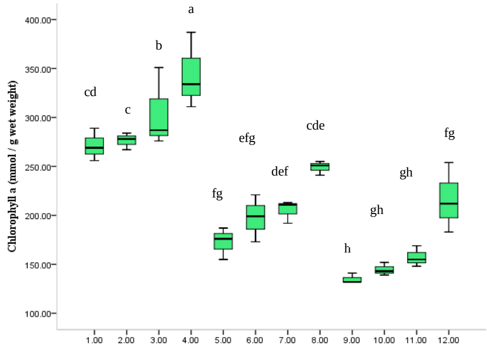

Figure 2. Changes in chlorophyll a by inoculation of barley under drought stress

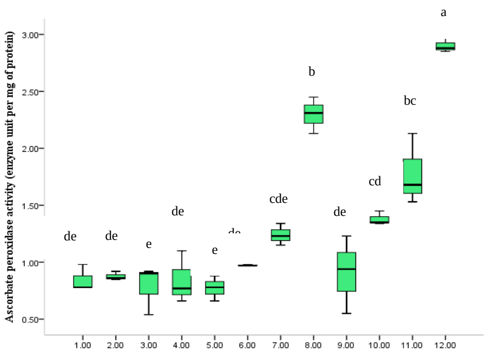

Figure 3. Changes in ascorbate peroxidase activity with barley inoculation under drought stress

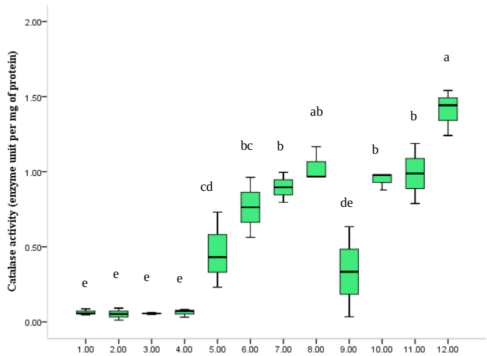

Figure 4. Changes in catalase activity with inoculation of barley under drought stress

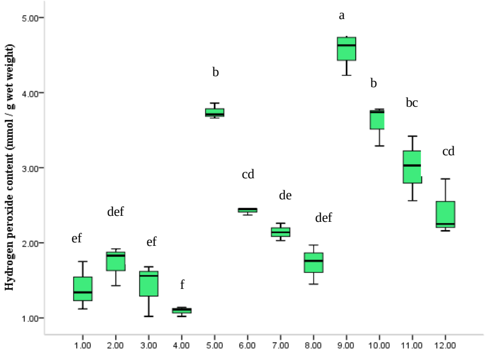

Figure 5. Changes in the amount of hydrogen peroxide with inoculation of barley under drought stress

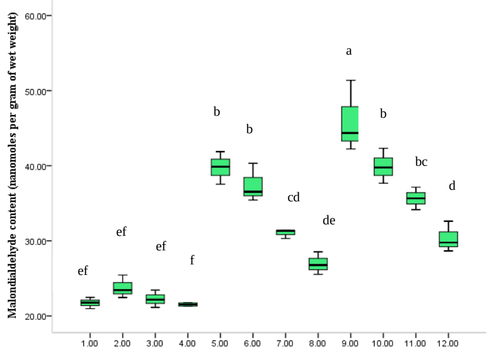

Figure 6. Changes in malondialdehyde content with barley inoculation under drought stress

The study results of the activity of superoxide dismutase, catalase and ascorbate can confirm this result.

The results of analysis of variance of the present study showed that the interaction between the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress was significant on malondialdehyde accumulation at the probability level of 1% (Table 2). By increasing water shortage, the accumulation of malondialdehyde inside plant cells increased, so that the highest amount of this matter was observed in irrigation treatment at 50% of field capacity and no use of fungi and bacteria (45.98 nmol / mg fresh weight) (Figure 6). On the one hand, using fungi and bacteria in irrigation treatment at 50% of field capacity, the amount of malondialdehyde accumulation was significantly reduced. The amount of this matter in the combined use of fungi and bacteria in irrigation treatment at 50% of field capacity was recorded at about 30.35 nmol / g fresh weight. In other words, only with the simultaneous use of fungi and bacteria in irrigation conditions in 50% of field capacity, the amount of malondialdehyde accumulation reduced by 33.9% (Figure 6). It should be noted that the lowest accumulation of malondialdehyde was recorded in irrigation treatment at 90% of field capacity using bacteria (21.01 nmol/mg fresh weight) (Figure 6). Malondialdehyde is a biomarker used to study the effects of environmental stresses on lipid peroxidation and membrane damage (Gawel *et al*., 2004). Damage to membranes is one of the destructive effects of reactive oxygen species. It should be noted that the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. in various ways can increase the tolerance of plants to drought stress (Amina and Hanan, 2011) and by preventing oxidative stress and reducing the damage caused by free radicals, increase the tolerance of plants to drought stress. For non-use of fungi and bacteria, one of the reactions that accelerates with the production of reactive oxygen species is the peroxidation of membrane lipids, which produces aldehydes such as malondialdehyde and products such as ethylene (Srivall and Khanna, 2004). The reduction in this compound is due to the activity of antioxidant enzymes. By increasing the activity of these enzymes, the amount of active oxygen produced under drought stress is controlled and the severity of damage to vital biomolecules and metabolic disorders is reduced. According to the results of the present study on the accumulation of hydrogen peroxide, it was found that the highest accumulation of this matter as one of the types of active oxygen was obtained from irrigation treatment at 50% of field capacity and no inoculation of fungi and bacteria that can confirm high amount of malondialdehyde accumulation in this treatment.

The results of analysis of variance of the present study showed that using endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress had a significant effect on plant proline content (Table 2).

We obtained the highest and lowest proline content (40.92 and 24.47 mg / g fresh weight, respectively) from the treatment of irrigation at 90% of field capacity and non-inoculation of fungi and bacteria and treatment of irrigation at 50% of field capacity and the combined use of bacteria and fungi (Table 3). The accumulation of proline is directly related to plant drought tolerance. In this regard, there are reports that proline modifies the negative impact of moisture stress on carbon stabilization and can moderate the reduction in rubisco activity under such conditions (Fendina *et al*., 1993). One of the evaluation indicators of plants under water shortage stress is the accumulation of proline in various plant organs (Zengin, 2006). The osmotic regulatory effects of proline on water balance and drought stress tolerance have been reported in various studies (Mohammadkhani and Heidary, 2008). Xu et al., 2017 found that colonization of roots with P. indica under drought stress increased maize leaf proline content. Camaille *et al*., 2021 showed that using bacteria, the content of proline in wheat increased significantly. It has also been reported that inoculation with Bacillus species increased proline content under drought stress in maize seedlings. This may be due to the high regulation of proline biosynthesis. Because proline helps maintain cellular water status and protect membranes and proteins from degradation. Ansary *et al*., (2012) also reported that drought stress increased leaf proline in maize, which is also found in plants inoculated with *P. fluorescens*. Although accumulation of proline during a short period after the end of stress helps the plant to regain its growth and therefore will have a positive effect on yield, but in various studies conducted under long-term drought stress, its effects on physiological and morphological properties were not significant and its accumulation will even have a negative impact on yield because it diverts plant photosynthetic resources to processes other than seed filling.

CONCLUSIONS

Today, using endophytic fungi has improved physiological and biochemical properties of various plants under drought stress. According to the results of the present study, inoculation with bacteria and fungi under drought stress improves leaf area, chlorophyll a, superoxide dismutase, Cu/Zn-SOD, Fe-SOD, Mn-SOD, ascorbate peroxidase isozymes, catalase peroxidase and glutathione reductase to the non-use of fungi and bacteria. So that we obtained the highest superoxide dismutase, Cu/Zn-SOD, Fe-SOD, Mn-SOD, ascorbate peroxidase, catalase, peroxidase and glutathione reductase isozymes from inoculation with bacteria and fungi with irrigation at 50% of the field capacity. Finally, it can be acknowledged that using fungi and bacteria through increasing the activity of the antioxidant system reduces the harmful effects of reactive oxygen species in Barley especially under stress. The result of these changes was a reduction in the content of hydrogen peroxide, malondialdehyde and proline compared to the treatment of non-use of fungi and bacteria. It should also be noted that inoculation with bacteria and fungi increased leaf area, chlorophyll b and carotenoids, the result of which can certainly be seen in modulating the growth of plants under stress and maintaining acceptable yield in these plants.

REFERENCES

Abid M, Ali S, Qi LK, Zahoor R, Tian Z, Jiang D, Dai T. (2018). Physiological and biochemical changes during drought and recovery periods at tillering and jointing stages in wheat (*Triticum aestivum* L.). Scientific reports, 8(1), 1-15.

Aebi H. 1984. Catalase in vitro Methods Enzymol, 105: 121–126.

Agami RA, Ghramh HA, Hasheem M. 2017. Seed inoculation with *Azospirillum lipoferum* alleviates the adverse effects of drought stress on wheat plants. Journal of Applied Botany and Food Quality, 90(1), 165-173.

Alscher RG, Erturk N, Heath LS. 2002. Role of superoxide dismutases (SODs) in controlling oxidative strese in plants. Journal of Experimental Botany. 53: 1331–1341.

Amina AA, Hanan HLL. 2011. Differential effects of paclobutrazol on water stress alleviation through electrolyte leakage, phytohormones, reduced glutathione and lipid peroxidation in some wheat genotypes (*Triticum aestivum* L.) grown in vitro. Rom. Biotech Lett. 6: 6710-6721.

Anjum F, Yaseen M, Rasool E, Wahid A, Anjum S. 2003. "Water stress in barley (*Hordeum vulgare* L.). II. Effect on chemical composition and chlorophyll contents." Pak. J. Agric. Sci 40(1-2): 45-49.

Ansary MH, Rahmani HA, Ardakani MR, Paknejad F, Habibi D, Mafakheri S. 2012. Effect of Pseudomonas fluorescent on proline and phytohormonal status of maize (*Zea mays* L.) under water deficit stress. Annals of Biological Research, 3(2), 1054-1062.

Asada K. 2000. The water–water cycle as alternative photon and electron sinks. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 355(1402), 1419-1431.

Ashraf M, Foolad MR. 2007. "Roles of glycine betaine and proline in improving plant abiotic stress resistance". Environmental and Experimental Botany. 59: 206–216.

Ashraf M, Harris PJC. 2004. "Potential biochemical indicators of salinity tolerance in plants" Plant Science 166: 3–16.

Ashraf M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnology advances, 27(1), 84-93.

Bacilio M, Rudriguez H, Moreno M, Hernandez JP, Bashan Y. 2004. "Mitigation of salt stress in wheat seedlings by a *gfp*-tagged *Azospirillum lipoferum*." Biol Fertil Soils 40:188–193.

Baisak R, Rana D, Acharya RBB, Kar M. 1994. Alternations in the activities of active oxygen scavenging of wheat leaves of Oryza sativa during drought. Journal of Experimental of Botany, 48: 2075-2085.

Baltruschat H, Fodor J, Harrach BD, Niemczyk E., Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarczinger I, Zuccaro A. 2008. Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol. 180: 501-510

Barassi CA, Ayrault G, Creus CM, Sueldo RJ, Sobrero MT. 2006. "Seed inoculation with *Azospirillum* mitigates NaCl effects on lettuce. " Sci. Hortic. (Amsterdam). 109: 8–14.

Barassi CA, Creus CM, Casanovas EM, Sueldo RJ. 2000. Could Azospirillummitigate abiotic stress effects in plants? Auburn University web site available at: http://www.ag.auburn.edu/argentina/pdfmanuscripts/barassi.pdf

Basak H, Demđr K, Kasim R, Okay FY. 2011. The effect of endo-mycorrhiza (VAM) treatment on growth of tomato seedling grown under saline conditions. African Journal of Agricultural Research, 6(11), 2532-2538.

Bashan Y, de-Bashan L. 2010. "How the plant growthpromoting bacterium *Azospirillum* promotes plant growth — a critical assessment. " Adv. Agron. 108: 77–136.

Bashan Y, Holguin G, de-Bashan L. 2004. "*Azospirillum*– plant relationships: physiological, molecular, agricultural, and environmental advances. " Can. J. Microbiol. 50: 521–577.

Biel W, Jacyno E. 2013. "Chemical composition and nutritive value of spring hulled barley varieties. " Bulg. J. Agric. Sci. 19: 721-727.

Breusegem FV, Vranova E, Dat JF, Inze D. 2001. The role of active oxygen species in plant signal transduction. Plant Science, 161:405-414.

Camaille M, Fabre N, Clément C, Ait Barka E. 2021. Advances in Wheat Physiology in Response to Drought and the Role of Plant Growth Promoting Rhizobacteria to Trigger Drought Tolerance. Microorganisms, 9(4), 687.

Casanovas EM, Barassi CA, Andrade FH, Sueldo RJ. 2003. *Azospirillum*-inoculated maize plant responses to irrigation restraints imposed during flowering. Cereal Res. Commun. 31:395–402.

Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis-Pinheiro M. 2012. Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. Genetics and molecular biology, 35(4), 1011-1019.

Chen LM, Lin CC, Kao CH. 2000. Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H2O2 level, and cell wall peroxidase activity in roots. Botanical Bulletin- Academia Sinica. 41: 99-103

Cossania CM, Slafer GA, Savin R. 2012. "Nitrogen and water use efficiencies of wheat and barley under a Mediterranean environment in Catalonia. " Field Crops Res. 128: 109-118.

Costa PH, Neto AD, Bezerra DA, Prisco JT. 2005. Antioxidant- enzymatic system of two sorghum genotypes differing in salt tolerance. Braz. J. Plant Physiol. 17(4):353-361.

Creus CM, Pereyra MA, Casanovas EM, Sueldo RJ, Barassi Ca. 2010. Plant growth-promoting effects of rhizobacteria on abiotic stressed plants. *Azospirillum*-Grasses model. *In* Plant science and biotechnology in South America: focus on Argentina 2. *Edited by* A. Di Benedetto. The Americas Journal of Plant Science and Biotechnology, 4: 49–59.

Creus CM, Sueldo RJ, Barassi CA. 2004. Water relations and yield in *Azospirillum*-inoculated wheat exposed to drought in the field. Can. J. Bot. 82: 273–281.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. Plant signaling & behavior, 3(3), 156-165.

Dimkpa C, Weinand T, Asch F. 2009. Plant–rhizobacteria interactions alleviate abiotic stress conditions. Plant, cell & environment, 32(12), 1682-1694.

Dismukes GC, Klimov VV, Baranov SV, Kozlov YN, DasGupta J, Tyryshkin A. 2001. The origin of atmospheric oxygen on Earth: The innovation of oxygenic photosynthesis. Proc Natl Acad Sci USA 98:2170–2175.

Dobbelaere S, Vanderleyden J, Okon Y. 2003. Plant growth promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. 22: 107–149.

Döbereiner J, Day JM. 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. *In* Proceeding of the 1st International Symposium of Nitrogen Fixation. *Edited by* E. Newton E and C.J. Nyman. Washington State University Press, Pullman, Washington. Pp: 518–538

Dolatabadi HK, Mohammadi Goltapeh E, Jaimand K, Rohani N. 2011. Effects of *Piriformospora indica* and *Sebacina vermifera* on growth and yield of essential oil in fennel (*Foeniculum vulgare*) under greenhouse conditions. J Basic Microbiol. 51:33–39.

FAO/WHO expert consultation - Bangkok, Thailand, FAO, Rome. " Chapter 16. (2009).;257- 270.

FAO/WHO. " Human vitamin and mineral requirements - Report of a joint

Fendina IS, Tsonev T, Guleva EL. 1993. The effect of pretreatment with praline on the responses of (*Pisum sativum* L.) to salt stress. Photosynthetica 29:521-527.

Fibach-Paldi S, Burdman S, Okon Y. 2012. Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense.* FEMS Microbiol. Lett. 326: 99–108.

Foyer CH, Shigeoka S. 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology*, 155, 1, 93–100.

Gawel S, Warda, M, Niedworok E, Wardas P. 2004. Malondialdehyde (MDA) as a lipid peroxidation marker. Wiad Lek. 57(9-10):453-5.

Giri B, Mukerji KG. 2004. Mycorrhizal inoculant alleviates salt stress in Sesbania aegyptiaca and Sesbania grandiflora under field conditions: evidence for reduced sodium and improved magnesium uptake. Mycorrhiza, 14(5), 307-312.

Gleick PH, Palaniappan M. 2010. Peak water limits to freshwater withdrawal and use. Proc Natl Acad Sci. 107:11155–11162.

Gregersen PL, Holm PB. 2007. Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). Plant Biotechnology Journal, 5(1), 192-206.

Gupta S, Schillaci M, Walker R, Smith PM, Watt M, Roessner U. 2021. Alleviation of salinity stress in plants by endophytic plant-fungal symbiosis: current knowledge, perspectives and future directions. Plant and Soil, 461(1), 219-244.

Gururani MA, Upadhyaya CP, Baskar V, Venkatesh J, Nookaraju A, Park SW. (2013). Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in Solanum tuberosum through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. Journal of plant growth regulation, 32(2), 245-258.

Harman GE, Doni F, Khadka RB, Uphoff N. 2021. Endophytic strains of Trichoderma increase plants' photosynthetic capability. Journal of applied microbiology, 130(2), 529-546.

Heidary Y, Moaveni P. 2009. Study of Drought stress on accumulation and proline among aba in different genotypes forage corn. Research Journal of Biological Sciences. 4:1121- 1124.

Hosseini F, Mosaddeghi MR, Dexter AR. 2017. Effect of the fungus *Piriformospora indica* on physiological characteristics and root morphology of wheat under combined drought and mechanical stresses. Plant Physiology and Biochemistry, 118, 107-120.

Kadian N, Yadav K, Aggarwal A. 2013. Significance of bioinoculants in promoting growth, nutrient uptake and yield of Cyamopsis tetragonoloba L. Taub.''. European journal of soil biology, 58, 66-72.

Karuppanapandian T, Manoharan K. 2008. Uptake and translocation of tri- and hexa-valent chromium and their effects on black gram (*Vigna mungo* L. Hepper cv. Co4) roots. J Plant Biol 51:192–201.

Karuppanapandian T, Sinha P, Haniya AK, Premkumar G. 2006b. Aluminium-induced changes in antioxidative enzyme activities, hydrogen peroxide content and cell wall peroxidase activity in green gram (*Vigna radiata* L. cv. Wilczek) roots. J Plant Biol 33:241–246.

Karuppanapandian T, Sinha P, Haniya AK, Premkumar G. 2006c. Chromium toxicity: Correlated with increased in degradation of photosynthetic pigments and total soluble protein and increased peroxidase activity in green gram (*Vigna radiata* L.) seedlings. J Swamy Bot-Cl 23:117–122

Karuppanapandian T, Sinha P, Hniya AMK, Mamoharan K. 2006a. Differential antioxidative responses of ascorbate-glutathione cycle enzymes and metabolites to chromium stress in green gram (*Vigna radiata* L. Wilczek) leaves. J Plant Biol 49:440–447

Karuppanapandian T, Wang HW, Prabakaran N, Jeyalakshmi K, Kwon M, Manoharan K, Kim W. 2011. 2,4-dichlorophenoxyacetic acid-induced leaf senescence in mung bean (*Vigna radiata* L. Wilczek) and senescence inhibition by co-treatment with silver nanoparticles. Plant Physiol Biochem 49:168–177.

Kim K, Wang MC, Ranjitkar S, Liu SH, Xu JC, Zomer RJ. 2017. Using leaf area index (LAI) to assess vegetation response to drought in Yunnan province of China. Journal of Mountain Science, 14(9), 1863-1872.

Kranner I, Beckett RP, Wornik S, Zorn M, Pfeifhofer HW. 2002. Revival of a resurrection plant correlates with its antioxidant status. The Plant Journal, 31(1), 13-24.

Kumar V, Sahai V, Bisaria VS. 2011. High-density spore production of Piriformospora indica, a plant growth-promoting endophyte, by optimization of nutritional and cultural parameters. Bioresour Technol 102:3169–3175.

Lee, S. H., Ahsan, N., Lee, K. W., Kim, D. H, Lee DG, Kwak SS, Lee BH. 2007. Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. Journal of plant physiology, 164(12), 1626-1638.

Li RH, Guo PG, Michael B, Stefania G, Salvatore C. 2006. Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. Agricultural Sciences in China, 5(10), 751-757.

Lisar SY, Motafakkerazad R, Mosharraf Hossain M, Rahman I. 2012. Water stress in plants: causes, effects and responses. In: Ismail, M.D., Mofizur, R., Hiroshi, H. (Eds.), Water Stress, Chapter1, pp1-14.

Liu F, Stützel H. 2004. Biomass partitioning, specific leaf area, and water use efficiency of vegetable amaranth (*Amaranthus* spp.) in response to drought stress. Scientia horticulturae, 102(1), 15-27.

Mahajan S, Tuteja N. 2005. Cold, salinity and drought stresses: an overview. Archives of biochemistry and biophysics, 444(2), 139-158.

Malinowski DP, Belesky D. 2000. Adaptations of Endophyte-Infected Cool-Season Grasses to Environmental Stresses: Mechanisms of Drought and Mineral Stress Tolerance. CROP SCIENCE. 40:923-940.

Manivannan P, Jaleel CA, Sankar B, Kishorekumar A, Somasundaram R, Lakshmanan GA, Panneerselvam R. 2007. Growth, biochemical modifications and proline metabolism in *Helianthus annuus* L. as induced by drought stress. Colloids and Surfaces B: Biointerfaces, 59(2), 141-149.

Manoharan K, Karuppanapandian T, Sinha P, Prasad R. 2005. Membrane degradation, accumulation of phosphatidic acid, stimulation of catalase activity and nuclear DNA fragmentation during 2,4-D-induced leaf senescence in mustard. J Plant Biol 48:394–403

Martin DL, Stegman EC, Fereres, E. 1990. Irrigation scheduling principles. IN: Management of Farm Irrigation Systems. American Society of Agricultural Engineers, St. Joseph, MI. 1990. p 155-203, 19 fig, 9 tab, 81 ref.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410

Mohammadkhani N, Heidari R. 2008. Effects of drought stress on soluble proteins in two maize varieties. Turkish Journal of Biology, 32(1), 23-30.

Oelmuller R, Sherameti R, Tripathi S, Varma A. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17.

Ommen OE, Donnelly A, Vanhoutvin S, Van Oijen M, Manderscheid R. 1999. Chlorophyll content of spring wheat flag leaves grown under elevated CO2 concentrations and other environmental stresses within the 'ESPACE-wheat'project. European Journal of Agronomy, 10(3-4), 197-203.

Orabi SA, Salman SR, Shalaby AF. 2010. Increasing resistance to oxidative damage in cucumber (*Cucumis sativus* L.) plants by exogenous application of salicylic acid and paclobutrazol. World Journal of Agricultural Sciences 6: 252-259.

Pereyra MA, Zalazar CA, Barassi CA. 2006. Root phospholipids in *Azospirillum* inoculated wheat seedling exposed to water stress. Plant Physiol. Biochem. 44:11–12.

Rostamza M, Chaichi MR, Jahansouz MR, Alimadadi A. 2011. Forage quality, water use and nitrogen utilization efficiencies of pearl millet (*Pennisetum americanum* L.) grown under different soil moisture and nitrogen levels. Agricultural Water Management 98: 1607-1614.

Sairam RK, Deshmukh PS, SAxena DC. 1998. Role of antioxidant systems in wheat genotypes tolerant to water stress. Biologia Plantarum. 41: 387-394.

Sairam RK, Rao KV, Srivastava GC. 2002. Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. Plant Sci. 163:1037-1046.

Sarker U, Oba S. 2018. Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of Amaranthus tricolor. Scientific reports, 8(1), 1-12.

Shao H, Liang ZS, Shao M. 2005. Changes of anti-oxidative enzymes and MDA content under soil water deficits among 10 wheat (*Triticum aestivum* L.) genotypes at maturation stage. Colloids Surfaces. 45: 7–13.

Sharma P, Ambuj BJ, Rama, SD, Pessarakli M. 2012. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. J. Bot. Vol. 26 Pp.

Sofo A, Scopa A, Nuzzaci M, Vitti A. 2015. Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. International Journal of Molecular Sciences, 16(6), 13561-13578.

Spaepen S, Vanderleyden J, Remans R. 2007. Indole-3-acetic acid in microbial and microorganism–plant signaling. FEMS Microbiol. Rev. 31(4): 425–448.

Srivall B, Khanna-Chopra R. 2004. The developing reproductive sink induces oxidative stress to mediate nitrogen mobilization during monocarpic senescence in wheat. Biochem and Biophy Res Commun. 325(1): 198- 202.

Stewart RRC, Bewley JD. 1980. Lipid peroxidation associated aging of soybean axes. Plant Physiology. 65: 245-248

Suzuki N, Mittler R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol. Plant. 126: 45-51.

Tan Y, Liang ZS, Hongbo HB, Du F. 2006. Effect of water deficits on the activity of anti-oxidative enzymes and osmoregulation among three different genotypes of Radix Astragali at graining stage. Colloids Surfaces. 49: 60-65.

Varma A, Bakshi M, Lou B, Hartmann A, Oelmueller R. 2012. Piriformospora indica: A novel plant growth-promoting mycorrhizal fungus. Agric. Res. 1:117–131.

Vellosillo T, Vicente J, Kulasekaran S, Hamberg M, Castresana C. 2010. Emerging complexity in reactive oxygen species production and signaling during the response of plants to pathogens. Plant Physiol 154(2):444–448.

Vranova E, Inze D, Breusegem FV. 2002. Signal transduction during oxidative stress. J Exp Bot 53(372):1227–1236.

Vurukonda SS KP, Vardharajula S, Shrivastava M, SkZ A. 2016. Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. Microbiological research, 184, 13-24.

Wang Y, Branicky R, Noë A, Hekimi S. 2018. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. Journal of Cell Biology, 217(6), 1915-1928.

Waszczak C, Carmody M, Kangajarvi J. 2018. Reactive oxygen speciesvin plant signaling. Annu. Rev. Plant Biol. 69:209-236.

Xu L, Wang A, Wang J, Wei Q, Zhang W. 2017. *Piriformospora indica* confers drought tolerance on *Zea mays* L. through enhancement of antioxidant activity and expression of drought-related genes. The Crop Journal, 5(3), 251-258.

Xu L, Wu C, Oelmüller R, Zhang W. 2018. Role of phytohormones in Piriformospora indica-induced growth promotion and stress tolerance in plants: more questions than answers. Frontiers in microbiology, 9, 1646.

Yaghoubian Y, Mohammadi Goltapeh E, Pirdashti H, Esfandiari E. 2014. Effect of Glomus mosseae and *Piriformospora indica* on Growth and Antioxidant Defense Responses of Wheat Plants Under Drought Stress. Agric Res. 3(3):239–245.

Yamazaki J, Ohashi A, Hashimoto Y, Negishi E, Kumagai S, Kubo T, Oikawa T, Maruta E, Kamimura Y. 2003. Effects of high light and low temperature during harsh winter on needle photodamage of Abies mariesii growing at the forest limit on Mt. Norikura in Central Japan. Plant Sci. 165:257-264.

Yong T, Zongsuo L, Hongbo S, Feng D. 2006. Effect of water deficits on the activity of anti-oxidative enzymes and osmoregulation among three different genotypes of Radix astragali at seedling stage. Colloids and Surfaces B: Biointerfaces 49: 60-65.

Yoshimura K, Yabuta Y, Ishikawa T, Shigeoka S. 2000. Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. Plant Physiology, 123(1): 223-234.

Zarea MJ, Hajinia S, Karimi N, Goltapeh EM, Rejali F, Varma A. 2012. Effect of *Piriformospora indica* and *Azospirillum* strains from saline or non-saline soil on mitigation of the effects of NaCl. Soil Biology and Biochemistry, 45, 139-146.

Zengin KF. 2006. The effects of Co2+ and Zn2+ on the contents of protein, abscisic acid, proline and chlorophyll in bean (Phaseolus vulgaris cv. Strike) seedlings. J. Environmental Biology. 27: 441-448.

Zur Y, Gitelson AA, Chivkunova OB, Merzlyak MN. 2000. The spectral contribution of carotenoids to light absorption and reflectance in green leaves. 2nd International Conference Geospatial Information in Agriculture and Forestry, Buena Vista, FL, 10-12 January, v. 2.

Leto, A., Hadžić, S., Vukotić, Dž., Salčinović, A. (2021): Effects of different Pb and Cd concentrations on bioavailability in some plant species. Agriculture and Forestry, 68 (1): 285-296. doi:10.17707/AgricultForest.68.1.18

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EFFECTS OF DIFFERENT Pb AND Cd CONCENTRATIONS ON BIOAVAILABILITY IN SOME PLANT SPECIES

SUMMARY

Our research aims to evaluate the effect of bunch load variation per unit area on The aim of this study was to examine the effect of different concentrations of heavy metals Pb and Cd on bioavailability in five plant species: nettle (*Urtica dioica* L.), spelt/dinkel wheat (*Triticum spelta* L.), spinach (*Spinacea oleracea* L.), phacelia (*Phacelia tanacetifolia* Benth.) and buckwheat (*Fagopyrum esculentum* Moench). These plant species were sown in containers with substrates contaminated with three different concentrations of Pb and Cd. After removal of the crop,the concentration of these metals was examined in the roots and aboveground organs of the plants. The results showed that with increasing concentration of Pb and Cd in the substrate, their concentration in plants increases, except in spelt where the highest concentration of Pb was recorded in the root of spelt grown on the substrate with the lowest concentration of Pb. The results of phytotranslocation potential showed that buckwheat is a suitable plant species for phytoextraction of both Pb and Cd with all three substrate variants, that is at all tested concentrations of both heavy metals.

Keywords: heavy metals, lead, cadmium, substrate, phytoextraction

INTRODUCTION

Heavy metals are among the most widespread pollutants in soil, water, and the environment. By polluting vast areas around the world, heavy metals are highly reactive and toxic, posing serious risks to human health and ecosystems (Raskin and Ensley, 2000; Wuana and Okieimen, 2011). Heavy metals cannot be degraded and can survive in the environment even after the removal of pollution sources (Babin-Fenske and Anand, 2010). The problem of accumulation of heavy metals in the soil has so far been solved by expensive, abrasive, chemical, and

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physical methods, which due to the lack of universal chemicals that would be used for all metals were not efficient, easily applied, and cost-effective (Radočaj et al., 2020).

In recent years, more and more attention is paid to the application of biological, less obstructive technologies in the domain of the new scientific discipline, the so-called phytoremediation. Numerous studies show that certain plant species have a huge genetic potential that enables efficient accumulation and removal of toxic heavy metals from the soil so that they can play an important role in the phytoremediation process (Jakovljević et al., 2016). To date, about 450 hyper accumulating types of heavy metals from 45 families have been identified (Verbruggen et al., 2009).

Regarding the frequent floods in BiH and the danger of soil contamination with heavy metals after the water withdrawal, attempts are being made to find an environmentally friendly method to clean the land (Ahmetović et al., 2020).

In this paper, the possibility of using plant species was investigated: nettle (*Urtica dioica* L.), spelta (*Triticum spelta* L.), spinach (*Spinacea oleracea* L.), phacelia (*Phacelia tanacetifolia* Benth.) and buckwheat (*Fagopyrum esculentum* Moench.) for remediation of contaminated soil with heavy metals: lead- Pb, cadmium - Cd. The selection of these plants for the experiment was made according to its adaptation to local climatic conditions, large production of green mass, depth to which the root penetrates, growth rate, ease of cultivation, ability to absorb large amounts of water and contaminants (heavy metals) and ability to remove toxins from the soil. The aim of this study was to define an effective and non-destructive method for the selection of native researched plant species and which plant organs show the largest phytoremediation potential based on their spontaneous recovery capacities. Phytoremediation using native species may be effective and efficient than its non-native counterparts, and it is ecologically safer, cheaper, aesthetically pleasing, socially acceptable and easier to cultivate (Heckenroth et al., 2016; Futughe et al., 2020).

Nettle (*Urtica dioica*) belongs to the hyperaccumulating type of heavy metals (Hartley, 2004) but its wider phytoremediation potential is neglected. Nettle, after four months of cultivation in soil contaminated with heavy metals, shows a phytoremediation capacity of 8% for Zn, Cd, and Pb (Viktorova et al., 2016).

Spelt (*Triticum spelta*) is a crop that better absorbs nutrients from the soil compared to common wheat. In the study of the impact of contaminated Cd soil on edible parts of the spelt/ dinkel wheat, a high translocation of Cd from the soil to the edible part of the plant was observed, although the concentration of Cd in the soil was below the threshold for agricultural land. (Radovanović et al., 2017).

Studies on the phytoremediation potential of spinach (*Spinacea oleracea*) show the accumulation of large amounts of heavy metals in tissues, but without visible signs of toxicity. Since the content of Cd and Pb in the edible part of the plant was above the safe limits, the research indicates environmental dangers that may arise when growing spinach for agricultural purposes in contaminated soil (Chatuverdi et al., 2019).

Studies of phytoremediation properties of buckwheat (*Fagopyrum esculentum*) on different types of soil contaminated with Cd or Pb show that plants growing in organic acidic soil show the highest sensitivity to Cd toxicity. Based on the translocation factor, we can conclude that buckwheat has a high phytostabilization potential with Pb, while for Cd it is low. This plant can be considered a candidate for phytostabilization in soils contaminated with Pb (Domaska et al., 2021).

This study investigated the possibilities and abilities of accumulation, conversion of heavy metals into insoluble forms and their removal from the soil by the five investigated plant species, and determining whether the concentration and availability of pollutants in soil decreased in an environmentally friendly way, thus creating opportunities for usage listed plants for remediation of contaminated lands.

MATERIAL AND METHODS

The experiment was set up in the greenhouse of the Agro-Mediterranean Faculty at the "Džemal Bijedić" University in Mostar in controlled conditions. The bioavailability of heavy metals Cd and Pb was tested on 5 plant cultures:

1.nettle (*Urtica dioica* L.)
2.spelt/ Dinkel wheat (*Triticum spelta* L.)
3.spinach (*Spinacea oleracea* L.)
4.phacelia (*Phacelia tanacetifolia* Benth.)
5.buckwheat (*Fagopyrum esculentum* Moench.).

The plants were sown in plastic containers measuring 10 x 10 cm, which were filled with the substrate Terra brill - soil for flowers, which is suitable for selected plant species. Each plant was sown separately in special containers with three different concentrations of Pb and containers with three different concentrations of Cd, three times, which is a total of 90 containers in the experiment (Figure 1).

Fig. 1. Growing plants on a substrate contaminated with heavy metals

The minimum concentration added to the substrate in 15 containers (3 containers for each plant) is the maximum allowed amount of Pb and Cd determined by the Law on Agricultural Land ('Official Gazette of F BiH' No. 52/09) and the Rulebook on determining permitted quantities of harmful and dangerous substances in the soil and methods of their testing ('Official Gazette of the F BiH' No. 72/09). In the next 15 containers, Pb was added in concentrations that were 50% higher than the maximum allowed, and in 15 other containers, Cd was added in concentrations that were 50% higher than the maximum allowed. In the remaining 15 containers, Pb was added in 100% higher concentrations than the maximum allowed and Cd in 100% higher concentrations than the maximum allowed. After removal of the crop, sampling of plant material was performed by manually separating the root from the stem of each plant culture. A total of 180 plant samples were obtained (30 samples from each variant). Analyses for Pb and Cd content of all 180 samples was performed by the method of Atomic Absorption Spectrophotometers. The analyzes were performed in the reference laboratory of the Federal Institute of Agriculture in Sarajevo. The obtained analyses results were statistically processed using the statistical program SPSS 22. The influence of plant species and concentration of heavy metals Pb and Cd in the substrate was determined by a two-factor analysis of variance, and the differences between plants and the substrate concentration ($p < 0.05$). Based on the obtained analysis results, phytoremediation and phytoaccumulation potential were calculated for each observed plant and each concentration of Pb and Cd.

Physico-chemical characteristics of the substrates used are shown in Table 1. (taken from declaration).

Table 1. Physio-chemical characteristics of the used substrates

White peat	Black peat	NPK-fertilizer g/m³	Trace elements	Salts mg/dm^3	N mg/dm^3	P_2O_5 mg/dm^3	K_2O mg/dm^3
25%	75%	1500	100 g/m³ + Wetting agent	1,2– 1,7	130– 290	140– 340	170– 390

Heavy metals Pb and Cd in different concentrations were added to the containers with the substrate. Experiment variants with concentrations of added Pb and Cd are shown in Table 2.

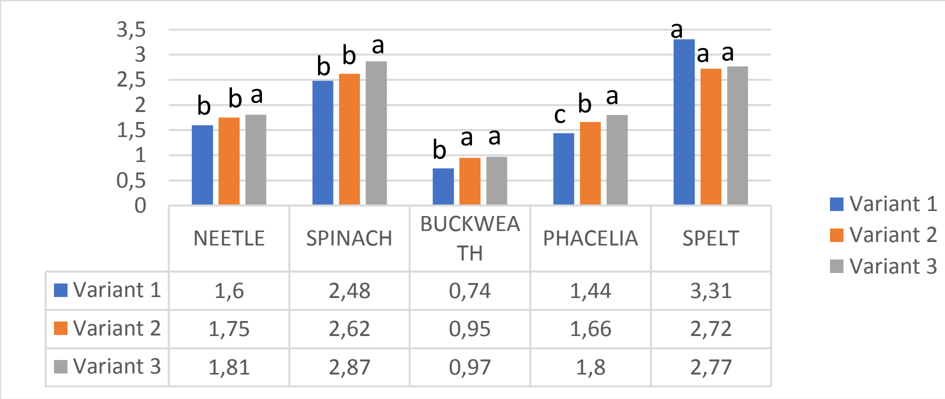
Table 2. Concentrations of metals in soil substrate in containers

Pb concentrations	Cd concentrations
80 mg/kg - Variant 1	1 mg/kg - Variant 1
120 mg/kg - Variant 2	1,5 mg/kg - Variant 2
160 mg/kg – Variant 3	2 mg/kg - Variant 3

RESULTS AND DISCUSSION

The results of research of Pb and Cd contents accumulated in the tested plant cultures, and depending on their concentration in the substrate during cultivation under controlled conditions, are presented in tabular form through

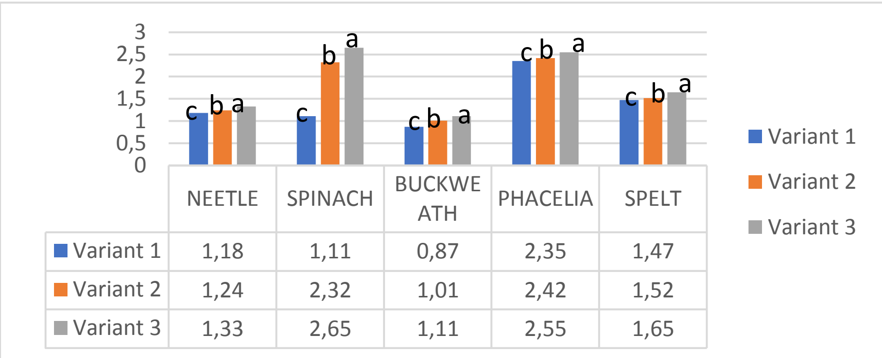
average values of heavy metal content and through statistical elements of all tested variants.
The lead content in the roots of the tested plants is shown in Graph 1.



	NEETLE	SPINACH	BUCKWEATH	PHACELIA	SPELT
Variant 1	1,6	2,48	0,74	1,44	3,31
Variant 2	1,75	2,62	0,95	1,66	2,72
Variant 3	1,81	2,87	0,97	1,8	2,77

Graph 1. Pb content (mg/kg) in the root of plants depending on the concentration in the substrate

The Pb content in the root of all observed plant species increased with increasing Pb concentration in the substrate. The exception to this is the spelled root whose root has the highest Pb concentration on the substrate with the lowest Pb concentration, which is also the highest Pb content in the root in Variant 1. In Variant 2 the highest content was recorded in the spelt compared to all other observed plants, while in Variant 3 the highest Pb content was recorded in spinach roots. The Pb content in the aboveground parts of the tested plants is shown in Graph 2.

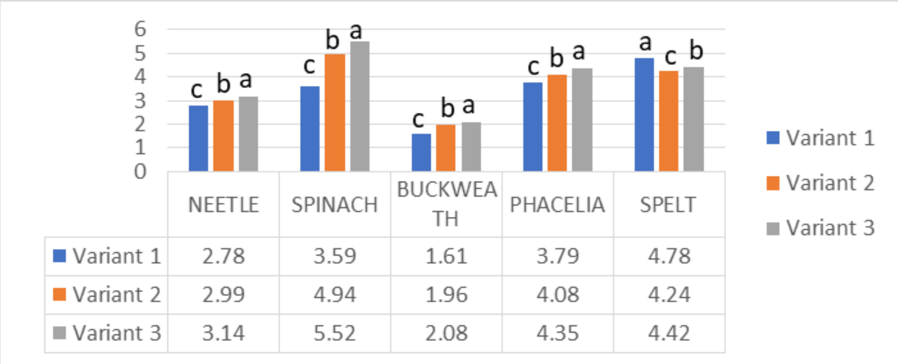


	NEETLE	SPINACH	BUCKWEATH	PHACELIA	SPELT
Variant 1	1,18	1,11	0,87	2,35	1,47
Variant 2	1,24	2,32	1,01	2,42	1,52
Variant 3	1,33	2,65	1,11	2,55	1,65

Graph 2. Pb content (mg/kg) in the aboveground part depending on the concentration in the substrate

The Pb content in the aboveground parts of the observed plants increased with increasing concentration in the substrate. In Variant 1 and Variant 2 it was the highest in phacelia plant, while in Variant 3 it was the highest in spinach.

Average total Pb content depending on the concentration in the substrate is shown in Graph 3.



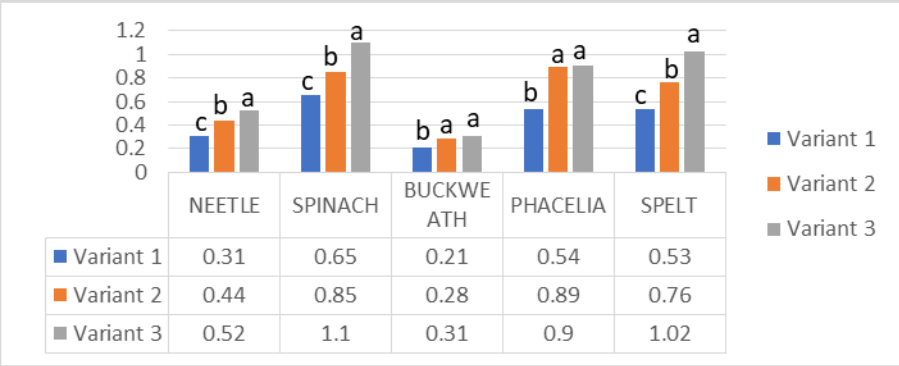
	NEETLE	SPINACH	BUCKWEA TH	PHACELIA	SPELT
Variant 1	2.78	3.59	1.61	3.79	4.78
Variant 2	2.99	4.94	1.96	4.08	4.24
Variant 3	3.14	5.52	2.08	4.35	4.42

Graph 3. Average total Pb content (mg/kg) depending on the concentration in the substrate

The total Pb content in all plants increased with increasing Pb concentration in the substrate except for spelt, where the total highest Pb content was recorded at the lowest Pb concentration in the substrate. For Pb, the normal concentration in plants is from 0.5 to 10 mg/kg of plant mass, and phytotoxic from 30 to 300 mg/kg (Baker and Brooks 1989), which means that in all tested plants the concentration of Pb was within normal limits. The threshold for Pb batteries was 100 mg/kg and for hyperaccumulators 1000 mg/kg (Boyd R.S. 2011), which means that, according to the obtained results, none of the tested plant species can be considered as either accumulator or a Pb hyperaccumulator.

The results of the analysis of variance showed that the plant species, and different substrate concentrations, and their interaction had a statistically significant effect on the total Pb content. The LSD test showed that spinach had a statistically significantly higher value compared to the other observed plant species, and that buckwheat had a statistically significantly lower total Pb content. Spelt had a higher value of total lead intake Pb compared to phacelia and nettle. Phacelia had a significantly higher total Pb content compared to nettle. All differences in the total Pb content in plants between substrates are significant. Significantly the highest Pb content in the aboveground parts of plants was measured on the substrate of Variant 3, and significantly the lowest value was registered on the substrate from Variant 1. The only deviation was registered in spelt with statistically higher total Pb content in the plant.

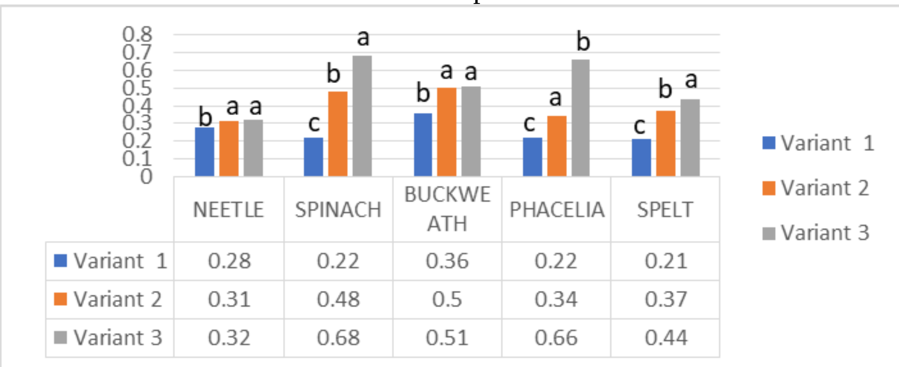
In all observed plant species, the Cd content in the root increased in parallel with the increase in the Cd concentration in the substrate. In Variant 1 and Variant 3, it was the highest in spinach, and in Variant 2 in phacelia. The content of Cd in the root of the examined plant species depending on its concentration in the substrate is shown in Graph 4.



	NEETLE	SPINACH	BUCKWE ATH	PHACELIA	SPELT
Variant 1	0.31	0.65	0.21	0.54	0.53
Variant 2	0.44	0.85	0.28	0.89	0.76
Variant 3	0.52	1.1	0.31	0.9	1.02

Graph 4. Cd content (mg/kg) in plant roots depending on the concentration in the substrate

The Cd content in the aboveground parts of the plant from variously contaminated substrates is shown in Graph 5 .



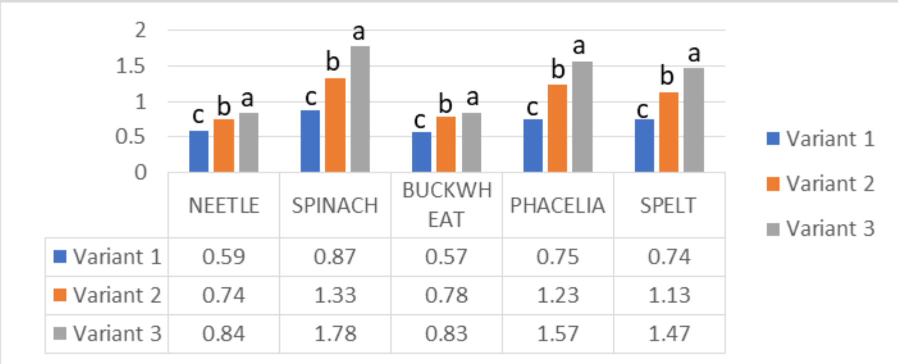
	NEETLE	SPINACH	BUCKWE ATH	PHACELIA	SPELT
Variant 1	0.28	0.22	0.36	0.22	0.21
Variant 2	0.31	0.48	0.5	0.34	0.37
Variant 3	0.32	0.68	0.51	0.66	0.44

Graph 5. Cd content (mg/kg) in the aboveground parts depending on the concentration in the substrate

The Cd content in the aboveground parts of all plants increased with increasing Cd concentration in the substrate. The highest in Variant 1 and Variant 2 was recorded in buckwheat, and in Variant 3 in spinach.

In all observed plant species, the total Cd content increased with increasing Cd concentration in the substrates. The highest total Cd content in all three substrate variants was recorded in spinach. The lowest was recorded in variant 1 in buckwheat, variant 2, and variant 3 in nettle. The normal amount of Cd in plants is 0.005 to 2 mg/kg, while phytotoxic ranges from 5 to 700 mg/kg (Baker, A. J. M., Brooks, R . R., 1989), so according to the results of this study, no plant is phytotoxic. The threshold for accumulators in Cd is 20, and for hyperaccumulators, it is 100 mg/kg (Boyd 2011), which means that, according to the obtained results, none of the tested plant species can be considered as an accumulator or hyperaccumulator of Cd. The results of the analysis of variance showed that the plant species, different concentrations of Cd in the substrate and their interaction had a statistically significant effect on the total Cd content. The

LSD test showed that spinach had a statistically significantly higher total Cd content compared to the other observed plant species and that nettle and buckwheat had a statistically significantly lower total Cd content. Phacelia had a significantly higher value compared to spelt. All differences in total Cd content among differently contaminated substrates are significant. Significantly the highest total Cd content was measured on the Variant 3 substrate, that is with the highest Cd concentration, and significantly the lowest value was registered on the Variant substrate, with the lowest Cd concentration. Average total Cd content in plants depending on the concentration in the substrate is shown in Graph 6.



Graph 6. Average total Cd content (mg/kg) in plants depending on the concentration in the substrate

Based on the value of phyto - accumulation potential, the ability of the plant to accumulate metals from the soil can be determined, and based on the value of the translocation potential, the ability of the plant to translocate the adopted metals from the roots to aboveground organs can be determined. Plants with this index equal to or greater than one are considered suitable for phyto-extraction of heavy metals from the soil. The values of phyto-translocation and phyto-accumulation potential are shown in Tables 3 and 4.

Based on the obtained results, it can be concluded that nettle is suitable for phyto-extraction of Pb if its concentration is 160 mg/kg. Buckwheat is suitable for phyto-extraction of both Pb and Cd at all concentrations. Phacelia is suitable for phyto-extraction at all Pb concentrations.

Research on the possibility of using native plants in the process of phytoremediation is often conducted due to the possibility of simpler and environmentally friendly ways of cleaning the soil from heavy metals.

In the study conducted in Western Serbia (Dimitrijević *et al.*, 2016) of bioaccumulation of heavy metals using nettle the results of investigation have shown that nettle has a tendency to accumulate Pb which may be used in phytoremediation of polluted soil which is in line with our research.

Table 3. Phyto-translocation potential values

PLANT SPECIES		Lead - Pb conc. mg/kg			Cadmium – Cd conc. mg/kg		
	Plant part	**80**	**120**	**160**	**1**	**1,5**	**2**
Neetle	Root	1,60	1,75	1,81	0,31	0,44	0,52
	Aboveground part	1,18	1,24	2,55	0,28	0,31	0,32
	TF index	**0,74**	**0,71**	**1,41**	**0,90**	**0,70**	**0,62**
Spinach	Root	2,48	2,62	2,87	0,65	0,85	1,10
	Aboveground part	1,11	2,32	2,65	0,22	0,48	0,68
	TF index	**0,45**	**0,89**	**0,92**	**0,34**	**0,56**	**0,62**
Buckwheat	Root	0,74	0,95	0,97	0,21	0,28	0,31
	Aboveground part	0,87	1,01	1,11	0,36	0,50	0,51
	TF index	**1,18**	**1,06**	**1,14**	**1,71**	**1,79**	**1,65**
Phacelia	Root	1,44	1,66	1,80	0,54	0,89	0,90
	Aboveground part	2,35	2,42	2,55	0,22	0,34	0,66
	TF index	**1,63**	**1,46**	**1,42**	**0,41**	**0,38**	**0,73**
Spelt	Root	3,31	2,72	2,77	0,53	0,76	1,02
	Abveground part	1,47	1,52	1,65	0,21	0,37	0,44
	TF index	**0,44**	**0,56**	**0,60**	**0,40**	**0,49**	**0,43**

Table 4. Phyto-accumulation potential values

PLANT SPECIES		Lead - Pb conc. mg/kg			Cadmium – Cd conc. mg/kg		
	Total plants/ substract	Variant 1	Variant2	Variant 3	Variant 1	Variant 2	Variant 3
Neetle	total	2.78	2.99	3.14	0.59	0.74	0.84
	substract	80	120	160	1	1,5	2
	TT index	**0.034**	**0.025**	**0.019**	**0.59**	**0.493**	**0.420**
Spinach	total	3.59	4.94	3.52	0.87	1.33	1.78
	substract	80	120	160	1	1,5	2
	TT index	**0.044**	**0.041**	**0.022**	**0.87**	**0.886**	**0.890**
Buckwheat	total	1.61	1.96	2.08	0.57	0.78	0.83
	substract	80	120	160	1	1,5	2
	TT index	**0.020**	**0.016**	**0.013**	**0.57**	**0.52**	**0.415**
Phacelia	total	3.79	4.08	4.35	0.75	1.23	1.57
	substract	80	120	160	1	1,5	2
	TT index	**0.047**	**0.009**	**0.027**	**0.75**	**0.82**	**0.785**
Spelt	total	4.78	4.24	4.42	0.74	1.13	1.47
	substract	80	120	160	1	1,5	2
	TT index	**0.059**	**0.035**	**0.028**	**0.740**	**0.750**	**0.735**

A pot study (Chaturvedi *et al.,* 2019) was conducted to assess the phytoremediation potential of Spinach plants along with their physiological and biochemical response when grown in soil contaminated with heavy metal(loid)s (HMs). Plants were grown under different doses of Pb, Cd and As; and their metal(loid) accumulation efficiency was studied upon harvest. Despite of accumulating high amount of HMs in tissues, no visible signs of toxicity were seen; and hence the efficient survival and defense mechanism shown by spinach plants conclude that they are a viable option to be used for phytoremediation of sites contaminated with Cd and Pb which is in line with our research.

Possibilities for phytoremediation of facelia have been very little researched and there are no results that would be comparable to our research

Buckwheat has relatively high biomass productivity is adapted to many areas of the world, therefore buckwheat is widely used for the phytoremediation process. After harvest, Cd and Zn concentrations of plant biomass and translocation factors for Zn and Cd were determined. Cadmium accumulation in biomass significantly increased in dose-dependent manner (Kaplan and Akan 2018). In the Tamara *et al.* (2005) experiment, the possibility of buckwheat to absorb haevy metals was investigated. In the investigation common buckwheat grown in Pb-contaminated soil was found to accumulate a large amount of Pb in its leaves, stem and roots, without significant damage. The results of both these studies correspond to the results of our research that buckwheat is suitable for phyto-extraction of Cd and Pb.

CONCLUSIONS

In the root of all tested plant species, the Pb content increased with its content in the substrate, except in spelled in which the highest Pb content in the root was on the substrate with the lowest Pb concentration. This also affected the results of the total Pb content in plants, so in spelled it was the highest on the substrate with the lowest Pb concentration. The results on the Pb content in the aboveground organs of all five plant species show that the Pb content increased with increasing concentration in the substrate. The content of Cd in both the root and aboveground organs, as well as in plants in all five examined plant species, grew with increasing concentration of Cd in the substrate. The highest total content in Cd in all Variants, i.e. at all concentrations in the substrate, was recorded in spinach. The values of phyto-accumulation potential in all tested plant species were less than 1.

The results of phyto-translocation potential showed that buckwheat is a suitable plant species for phyto-extraction of both Pb and Cd with all three substrate variants, i.e. at all tested concentrations of both heavy metals. Nettle is suitable for phyto-extraction of Pb at its extremely high **c**oncentration in the substrate, 160 mg/kg. Phacelia is suitable for phyto-extraction of Pb at all Pb concentrations in the substrate.

REFERENCES

Ahmetović, M., Keran, H., Šestan, I., Odobašić, A., Čanić, A., Junuzović, H., Hrnjić, N. (2020). Influence of the Spreča River Flooding on Individual Physicochemical Parameters of Soil. International Journal for Research in Applied Sciences and Biotechnology. Volume-7, Issue-3 (May 2020)

Babin-Fenske, J. J., Anand, M. (2010). Terrestrial insect communities and the restoration of an industrially perturbed landscape: assessing success and surrogacy. Restoration Ecology, 18: 73–84

Baker, A. J. M., Brooks, R. R. (1989) Terrestrial higher plants which hyper accumulate metallic elements – Review of their distribution, ecology and phytochemistry// Biorecovery, 1: 81-126

Boyd R. S. (2011). The defense hypothesis of elemental hyperaccumulation: status, challenges and new directions. Plant Soil 293: 153-176

Chatuverdi, R., J., Favas, P., Pratas, J., Varun, M., S., Manoj, P. (2019). Metal(loid) induced toxicity and defense mechanisms in *Spinacia olerace*a L. Ecological hazard and Prospects for phytoremediation. Ecotoxicology and Environmental Safety. Volume 183. 109570

Dimitrijević, V. D., Krstić, N. S., Stanković, M. N., Arsić, I., Nikolić, R.S. (2016). Biometal and heavy metal content in the soil-nettle (*Urtica dioica* L.) system from different localities in Serbia Advanced technologies 5(1) (2016) 17-22

Domanska, J., Leszczyńska, D., Badora, A. (2021). The Possibilities of Using Common Buckwheat in Phytoremediation of Mineral and Organic Soils Contaminated with Cd or Pb. Agriculture, 11, 562

Futughe, A. E., Purchase, D., Jones, H. (2020). Phytoremediation Using Native Plants. In: Shmaefsky B. (eds) Phytoremediation. Concepts and Strategies in Plant Sciences. pp 285-327 Springer, Cham.

Hartley, L. (2004). Characterization of a Heavy Metal Contaminated Soil in Ohio for a Phytoremediation Project: University of Toledo

Heckenroth, A., Rabier, J., Dutoit, T., Torre, F., Prudent, P., Laffont-Schwob, I. (2016). Selection of native plants with phytoremediation potential for highly contaminated Mediterranean soil restoration: Tools for a non-destructive and integrative approach. Journal of Environmental Management 183 (2016) 850e863 851

Jakovljević, T., Radojčić Redovniković I., Laslo, A. (2016). Phytoremediation of heavy metals - Applications and experiences in Croatia. Review paper ISSN 0351-9465, E-ISSN 2466-2585 UDC:628.168:632.122.23 doi:10.5937/ZasMat1603496J. Zaštita Materijala 57 (3): 496 - 501

Kaplan, N. O., Akan, A. (2018). The determination of Cd and Zn phytoremediation potential of buckwheat (*Fagopyrum esculentum*), Desalination and Water Treatment 133:146-152

Law on Agricultural Land, Official Gazette of the F BiH, no. 52/09/-Law on agricultural land, Official Gazette F BIH, no. 52/09

Radočaj, D., Velić, N., Jurišić, M., Merdić, E. (2020). The remediation of agricultural land contaminated by heavy metals. Poljoprivreda/Agriculture 26:2020 (2): 30-42

Radovanović, V., Životić, Lj., Žarković, B., Đorđević, A. (2017). Siot-to-plant bio-accumulation factor as indicator of trace metal implementation into the food chain. Carpathian Journal of Earth and Environmental Sciences, July 2017, Vol. 12, No. 2: 457 – 462

Raskin, I., Ensley, B.D. (2000) Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment. Open Journal of Ecology, Vol.5 No.8, 53-70

Rulebook on Determining Permitted Quantities of Harmful and Dangerous Substances in Soil and Methods of Their Testing, Official Gazette of the F BiH, no. 72/09

Tamara, H., Honda, M., Sato, T., Kamachi, H, (2005). Pb Hyperaccumulation and Tolerance in Common Buckwheat (*Fagopyrum esculentum* Moench), Journal of Plant Research 118(5):355-9, 10.1007/s10265-005-0229-z

Verbruggen, N., Hermans, C., Schat, H. (2009). Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*. 181(4):759–76. 10.1111/j.1469-8137.2008.02748.

Viktorova, J., Jandova, Z., Madlenakova, M., Prouzova, P., Bartunek, V., Vrchotova, B., Lovecka, P., Musilova, L., Macek, T. (2016). Native Phytoremediation Potential of *Urtica dioica* for Removal of PCBs and Heavy Metals Can Be Improved by Genetic Manipulations Using Constitutive CaMV 35S Promoter. PLoS One 11 (12), e0167927

Wuana, R.A., Okieimen, F.E. (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. ISRN Ecology, 1–20

Ikanović, J., Popović, D., Popović, V., Jaćimović G., Đurović, I., Kolarić, Lj., Ćosić, M., Rakaščan, N. (2022): Analysis of Genotype-by-Year interaction for Secale cereale L. productive traits and circular economy. Agriculture and Forestry, 68(1):297-319. doi:10.17707/AgricultForest.68.1.19

DOI: 10.17707/AgricultForest.68.1.19

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ANALYSIS OF GENOTYPE-BY-YEAR INTERACTION FOR *Secale cereale* L. PRODUCTIVE TRAITS AND CIRCULAR ECONOMY

SUMMARY

The circular economy offers a new product-waste-product model, in this case obtaining biofuels from rye biomass. The circular economy introduces a new product design, which will enable its functionality for a longer period of use. *Secale cereale* L. is an economically important crop for food, feed and bioenergy. The objective of this study was to estimate productivity of rye genotypes and the possibility of obtaining biogas from rye biomass during two growing seasons, 2019-2020. The aim of this study was to examine the influence of year and genotype on rye productivity parameters, biogas, methane yield, methane proportion, and the possibility of using rye as an alternative fuel in Serbia. The influence of the year and genotypes on the parameters of rye productivity, biogas and methane yield, methane content and the possibility of using rye as an alternative fuel in Serbia was investigated. Genotype and year × genotype interaction had a statistically significant effect on biogas yield, methane yield and methane content in the studied rye genotypes. Genotype G1 had the mean of green biomass yield (25.73 t ha^{-1}) significantly higher compared to genotype G2 (23.75 t ha^{-1}) in both years of experiment. Green biomass yield (24.11 t ha^{-1}) was better in 2019 compared to 2020. Biogas yield varied from 260.57 m^{3} ha^{-1} (genotype G1) to 214.58 m^{3} ha^{-1} (genotype G2). Biogas yield were better in 2019

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(237.85 $m^3 ha^{-1}$) compared to 2020 (237.30 $m^3 ha^{-1}$). A positive statistically highly significant correlation was attained between the green biomass yield and the length of the spikes (0.82**), green biomass yield and biogas yield (0.93**), green biomass yield and methane content (0.90**).

Keywords: rye; biomass, biogas yield, G×Y interaction; circular economy.

INTRODUCTION

After wheat, rye is the second most important raw material for bread and bakery products, and it is one of the most excellent sources of dietary fibres and bioactive compounds. Rye is utilised in more other food products as well, such as breakfast cereals, porridges, pasta, snack products, etc. Recent scientific research is focused on studying the possible health benefits and the potential of rye in the development of novel food products but also the possibility of using it for energy purposes. Rye (*Secale cereale* L.) is a smal grain cereal perfectly adapted to different agroecological conditions, so that it has a very large area of distribution. Areas under rye are significantly reducing in the world every year. In 2018, rye was grown on 4,403,020 ha in the world and on about 5,000 ha in Serbia. The main product is grain, which is mostly used for bread and bakery products and in the industry for the production of alcoholic beverages (Jordanovska *et al.,* 2018). Rye is main source of starch and energy. Rye grain contains numerous nutritional components such as proteins, fats, vitamins (B complex), dietary fibre and phytochemicals. Improving drought tolerance has always been an important objective in many crop improvement programs and is becoming more important as one way of adapting crops to climate changes (Belitz *et al.*, 2009; Lakew *et al.*, 2018). Nowadays, a larger number of hybrids are grown worldwide since they are more tolerant to drought in all phenophases (Ikanović *et al.,* 2013). In the past decades, many countries, under strong pressure to improve energy security from the aspect of environmental protection, but also to reduce dependence on imports, have begun to develop programs for the production of alternative biofuels (methane, ethanol and biodiesel) from plant products. Initially, the main crop products grain and fodder biomass were used for this purpose, while more recently systems for the use of waste of biological origin have been developed, with special emphasis on secondary-alternative-crop products and forest products (Janković *et al.*, 2019).

Circular economy is an "instrument" for the realization of sustainable development goals and implies long-term investment in raw material and energy efficiency, with reduction of harmful emissions, replacement of fossil fuels with renewable sources and production and trade in sustainable products, thus closing the circle "product-waste-product". Thanks to the development of new technologies for processing bio-waste into energy sources, the growth rate of the use of alternative fuels is growing significantly. According to estimates by energy experts, it is about 15% per year in highly developed countries. During the 21st century, population growth will be a big problem in finding solutions to provide the necessary amounts of food, but also energy, since the reserves of basic energy sources, which are fossil fuels, are limited. According to the forecasts of experts in these fields, food and energy consumption will double by 2050 (Popović *et al.*, 2020a; Rakaščan *et al.*, 2021).

By improving the technological process of obtaining biofuels from secondary products, energy sources would be obtained with much wider application. The advantage of these energy sources is the fact that they come from renewable sources, which significantly reduces the dependence on the import of fossil fuels, which a large number of countries do not have. Another positive effect would be significantly lower emissions of harmful gases into the atmosphere. By burning biofuels, all the carbon dioxide that goes into the atmosphere would be used by plants for photosynthesis processes during the year, and at the same time they release oxygen. The amounts of other harmful gases released by burning of these alternative fuels are also much lower than of fossil fuels. The combustion of these alternative fuels are also far less than from fossil fuels. Biomass of secondary products, which would be used to produce biofuels, is one of the ways in which countries could meet their obligations under the Kyoto Protocol on Climate Change, because, as a whole, they would reduce greenhouse gas emissions and the greenhouse effect, as a fundamental factor in global temperature rise (Ikanović *et al.*, 2013; 2018).

During the anaerobic digestion process, biogas is produced together with a valuable residual stream known as the digestate. Therefore, increasing demand for biogas-based energy generation will generate a significant increase in the annual volumes of digestate generated. Recycling the digestate back to soil and therefore valuable nutrients such as nitrogen, potassium, phosphorus and organic carbon for plants is the circular economy concept case (Provenzano *et al.*, 2018). Anaerobic digestion is a good example of a closed loop process as the biogas is produced from the volatile matter fraction of various biodegradable feedstock streams such as animal slurry, manure or agricultural waste biomasses and the valuable nutrients available in the digestate are recycled back to the soil. Economic and environmental sustainability is challenged by two major factors: by the distance and feedstock quantity used for biogas production and the amount of digestate generated during the anaerobic digestion process in each biogas power plant. The leading strategy for a circular economy-based digestate management approach is still in its immature phase (Peng and Pivato, 2017).

In addition to economic problems, there are also problems of environmental protection, because the increasing use of fossil fuels significantly increases the amount of harmful gases in the atmosphere. The consequence of increasing the concentration of these gases in the atmosphere (especially carbon dioxide) affects climate change, which is manifested by global warming of the planet due to the greenhouse effect.

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 clear and they insist that these secondary products be returned to the soil by ploughing or as manure, which has a far greater importance on soil fertility and further plant production. Finding the optimal solution for the use of grain straw should be the subject of further research (Collins *et al.*, 2014).

Rye has relatively drought tolerant compared to other cereal crops. Rye quality is very different from year to year (Đekić *et al.*, 2017; Đurić *et al.*, 2021). Examining the influence of genotype and environment on grain and quality traits, in a study with 19 different hybrid and population varieties grown for different years, found that variation in grain yield and protein concentration was mainly due to genotypes, but that thousand grain mass and dietary fiber concentration was more strongly influenced by harvest year than by genotype (Jordanovska *et al.*, 2018).

Grain yield is a function of genotype, environment and genotype x environment interaction (GEI) (Kota *et al.*, 2013; Djuric *et al.*, 2018; Đekić *et al.*, 2018; Kartina *et al.*, 2019; Amzeri *et al.*, 2020; Luković *et al.*, 2020; Khadka *et al.*, 2020; Rajičić *et al.*, 2021). An understanding of environmental and genotypic causes of GEI is important at all stages of crop improvement as they have a bearing on parent selection, selection based on yield as well as cultivar adaptation. GEI studies thus provide a basis for selection of genotypes that are suitable for general or specific cultivation; they also provide information about the effect of environment on cultivar performance (Khan *et al.*, 2007). The presence of genotype by environment (G × E) interaction is a major concern to rye breeders, since large interactions can reduce gains from selection and complicate identification of superior cultivars.

The objectives of our study are: a) to evaluate the influence of genotypes and environment on variation of productivity traits, b) to investigate correlations between traits, c) to to evaluate rye production in divergent years and assess the possibility of using rye as an energy crop and d) to point out the importance of the circular economy. This study suggests that the stability analysis may contribute to additional information on the performance of new rye selections prior to release for commercial cultivation and may increase the effictiveness of cultivar development programs.

MATERIAL AND METHODS

The experiment was conducted in Ilandza, Serbia, for two consecutive years (2018/2019 and 2019/2020). The trials were conducted according to a randomized block system in three replications with genotypes: G1- Propower (KWS) and G2- NS Savo (Institute of Field and Vegetable Crops, Novi Sad). Elementary plots were 10 m^2. The genotype G1 is energy while genotype G2 for the grain. During the experiment, the standard cultivation technology for rye production was applied. Preceding crop was soybean. At the pre-sowing preparation, NPK nutrients (350 kg ha^{-1}) were introduced. Sowing was 10/21/2018 and 10/26/2019 with cereal seeder. During the vegetation period, three time mechanical crop care measures were applied. Mowing was performed at waxy ripeness and then samples were taken, from each elementary plot, for the analysis of the morphological productive parameters. After the morphological parameters were measured, each of rye genotypes was separately cut and placed in trench silos, and covered for 40 days. After that the silage was placed in a fermenter and biogas was obtained from it.

Rye is sown until late September or early October. Rye tolerates temperatures very well from minus 25 degrees. It has a higher tolerance to diseases than wheat. For feeding new genotypes of rye 100-110 kg of nitrogen per hectare is needed, 60-80 kilograms of phosphorus and 40-60 kilograms of potassium. New generation genotypes achieve a yield of 7 to 8.5 tons per hectare. Rye is grown for grain and for silage but also for bioenergy. Silage is done in April, while the rye is still green. Rye then has a lot of protein quality livestock feed was obtained. Haylage yields are about 24,000 kilograms per hectare. Energy rye hybrids give higher biomass yield. The rye harvest for bioenergy is done around the middle of May (Glamočlija *et al.,* 2015; Lakić *et al.,* 2018). Rye by-products (biomass) is a good raw material for the production of alternative fuels.

Genotype G1 was selected for biogas production while genotype G2 was selected for grain and has high quality grains for various applications. Commonly in breeding for drought tolerance, grain yield is the basis for selection, but it is a complex, late-stage trait, affected by many factors aside from drought. For successful grain production, selection genotypes G2 is recommended, while for biogas production is recommended to the G1 genotype. If the goal of selection is to obtain a genotype for biogas production selection should focus on obtaining higher plants genotypes that is to obtain genotypes with higher biomass production.

Meteorological data

Weather conditions have a significant influence on biomass production and plant yield (Lakić *et al.,* 2018; 2020; Terzić *et al.,* 2019; Popović *et al.,* 2020a; 2020b; Rajičić *et al.,* 2020; Ljubičić *et al.,* 2021). This experiment was conducted for two years in Ilandza (45° 10′ 06″ N; 20° 55′ 06″ E, 59 m above sea level), on a sandy chernozem soil, in the municipality of Alibunar, in the South Banat district of Serbia.

During the vegetation period, the two years were significantly different. The total amount of precipitation was 484.3 mm in 2018/2019 and 538.3 mm in 2019/2020 (Table 1).

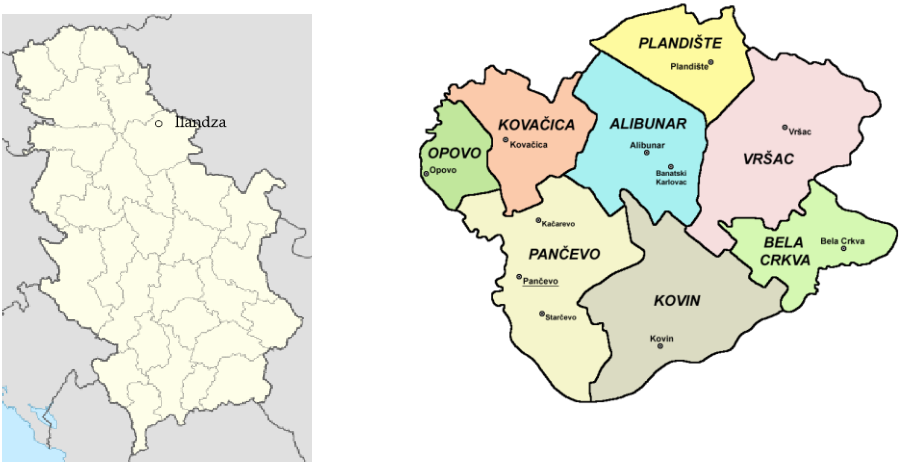
The average temperatures were 9.5°C in 2018/2019 and 9.6°C in 2019/2020. The total precipitation in 2018/2019 was lower by 54 mm compared to 2019/2020 and by 40 mm compared to the long-term period (Tab. 1).

Soil Analysis

The chemical analysis was performed of agrochemical characteristics of the soil in the municipality of Ilandza, on sandy chernozem soil, (Map 1a, 1b).

Table 1. Average temperature and total precipitation during the vegetation period in 2018/2019 and 2019/2020, Alibunar meteorological station

Parameter	Mounts								
Year	**10.**	**11.**	**12.**	**1.**	**2.**	**3.**	**4.**	**5.**	**Average**
Mean monthly temperature									
2018/2019	11.2	8.6	2.8	-0.2	3.2	9.5	11.0	17.2	9.5
2019/2020	11.5	9.0	2.0	0.3	3.1	8.5	11.9	18.0	9.6
Long term	12.6	7.5	2.0	0.8	2.6	7.6	13.0	18.0	9.6
Monthly precipitation sums									
2018/2019	36.2	41.6	33.2	47.2	20.3	32.6	67.2	90.0	484.3
2019/2020	48.3	52.4	62.1	48.3	39.2	52.0	72.1	93.4	538.3
Long term	48.8	52.0	45.8	51.0	55.2	55.8	48.9	82.3	524.0



(a) (b)

Map 1. Locality of Ilandza on the map of Serbia (a); Municipality of Alibunar, South Banat district, Serbia (b).

Soil samples were collected at 2018. Composite soil sample (0–30 cm depth) was a combination of five subsamples (one sample from each corner and one from the center of a 10 m^2). The following soil properties were measured according to standard methods: $CaCO_3$, soil pH, total nitrogen (TN), available phosphorus (AP), available potassium (AK) (according to Bogdanović and Ubavić, 1995). Analysis of the results obtained have shown that pH in H_2O is of weakly-alkaline (7.9) and pH in KCl was of neutral reaction (6.9), content of $CaCO_3$ was 2.1%, total nitrogen is 0.2% and very low content of available P_2O_5 and K_2O (0.6 mg/100 g and 0.4 mg/100 g of soil).

Biogas and methane production

Biogas from biomass is one of the possibilities of renewable energy production to reduce greenhouse gas emissions. The biogas yield (BGY) was determined by analysis of biomass in the laboratory of the Faculty of Engineering in Novi Sad, according to the method of VDI 4630 and was converted to Nm^3t^{-1} (Pham *et al.,* 2013). Energy crops are grown for the purpose of bioenergy production. Biomass now makes up the largest potential of agricultural residues, it is a versatile energy source that can substitute fossil energy in the energy sectors electricity. Biogas has an important role in the field of environmental conservation by mitigating global warming and conserving fossil fuel. The production of biogas from biomass or waste is one way to reduce both the consumption of crude oil and environmental pollution (Rakaščan *et al.,* 2021; Popović *et al.,* 2020a; Dražić *et al.,* 2021). The composition of biogas depends on the composition of the parent feedstock, its physical and chemical properties are similar to those of conventional diesel. Biogas is considered safe for the environment, showing insignificant contribution of carbon dioxide and particulate emissions (Zhu, 2018; Milanović *et al.,* 2020; Popović *et al.,* 2020a; Rakaščan *et al.,* 2021). Biogas is produced by the process of anaerobic digestion or fermentation. Anaerobic digestion is an established technology, we used it to treat a biomass - organic wastes. It is a biological process in which organic carbon, by oxides-reduction processes, is converted to the highest oxidation rate (CH_4), Table 10. This process takes place in the absence of oxygen and is catalyzed by many microorganisms (Cakić and Stamenković, 2009).

Table 2. Biogas components produced in anaerobic biogas reactors.

Compound	Methane	Carbon dioxide	Nitrogen	Hydrogen	Hydrogen sulfide	Oxigen
Formula	CH_4	CO_2	N_2	H_2	H_2S	O_2
Percentage by volume, %	50-70	25-50	0-10	0-1	0.1-0.5	0-0.5
Source: www.kolumbus.fi.						

Plants biomass can be used to produce briquettes and pellets, solid fuels suitable for use in smaller boiler plants, for example for heating residential buildings. It can also be used to produce liquid biofuels (ethanol) because it has high amounts of carbohydrates. The_technological process of processing into ethanol is carried out in stages. In the first phase, the biomass is chopped and treated with sodium hydroxide to break down the lignin, then, it is hydrolyzing by ferments that break down the complex sugars to hexoses. During the fermentation process, the hexose sugars are converted into ethyl alcohol by the glucoamylase ferment, releasing carbon dioxide ($C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$). In the distillation process, ethanol is separated from other by-products. Approximately 2 kg of glucose is required to obtain 1 kg of ethanol (Richards *et al.,* 1994; Milanović *et al.,* 2020). The estimation of G, E and G×E interactions (or GEI) ensures valid recommendations of suitable varieties able to overcome the pressure due to variable occurring conditions. The determination of GEI factors helps geneticists in their breeding programs to shift the selection toward varieties suited for wide environments or specific to certain niches. The production success was cultivar-dependent and the pedoclimatic conditions as essential factors in determining yield (Egea-Gilabert *et al.,* 2021).

The weekly methane production was calculated by multiplying the mass of individual substrate fed into the full-scale digesters on a basis with the properties of the different substrates determined in the laboratory. The obtained methane production of the substrates were added up supposing additivity without synergistic or inhibitory effects. The methane production was calculated as follows:

$P=\sum Qi\times TSi\times VSi\times BMPi[Nm^3CH_4\ week^{-1}]$

where Q is the mass of substrate fed into the digesters per week [tons], TS is the total solids content of the substrate [%], VS is the volatile solids content of the substrate [%], and BMP is the biomethane potential of the substrate [$Nm^3CH_4\ tVS^{-1}$]. The data on the mass of substrates fed per week were provided by the operators of the two full-scale AD plants (Holliger *et al.,* 2017).

Statistical Analysis

The experimental data obtained were analyzed by descriptive and analytical statistics, with the statistical package STATISTICA 12 for Windows (StatSoft). Testing the significance of the differences between the calculated mean values of the examined factors (Genotype and Years) was done by using a two-factor model of variance analysis. All significance ratings were derived from the LSD test for a significance level of 0.05% and 0.01%. The relative dependence between the tested parameters for rye was determined by the method of correlation analysis (Pearson's correlation coefficients), and the obtained coefficients tested by t-test for significance level of 0.05% and 0.01%. The obtained results are presented in Tables 3-11 and Figures 1-6.

RESULTS AND DISCUSSION

Plant height

The results showed that there was a significant difference between rye genotypes for plant height trait at 0.05% level in two years, Tab. 3, Fig. 1. There was a significant difference for the mean of plant height between two years. Genotype G1 had a statistically significantly higher studied parameter in 2019/2020 compared to genotype G2 in 2018/2019 (130 cm) and 2019/2020 (134.00 cm) and compared to genotype G1 in 2018/2019 (129.33 cm) (Table 3, Figures 1a, 1b). There were no statistically significant differences between the studied genotypes for the studied parameter, $p>0.05$. The genotype G1 had plants height of 135.17 cm on average in both trial years, while the height of plants for genotype G2 was 132 cm, Table 3.

Based on the analysis of variance, it can be concluded that there are highly significant differences in rye plants height between tested year ($F_{exp}=6.330^{*}$) and no significant differences at studied genotypes (Table 4).

Table 3. Productivity parameters of rye genotypes, Serbia, 2018/2019-2019/2020.

Parameter	**Genotype**	**Plant height, cm**	**Spike length, cm**	**Green biomass yield, t ha^{-1}**
2018/2019	G1	129.33±10.06	13.66±0.57	25.08±0.67
2019/2020		141.00±1.00	14.10±0.17	25.73±0.15
Average		135.16±9.04	13.88±0.44	25.41±0.56
2018/2019	G2	130.00±1.00	11.66±1.03	23.13±0.25
2019/2020		134.00±3.60	11.67±1.52	21.77±0.25
Average		132.00±3.22	11.67±0.57	22.45±0.78
Average 2018/19		129.66±6.40	12.66±1.50	24.11±1.16
Average 2019/20		137.50±4.50	12.88±1.38	23.75±2.18
Average 2018/19-2019/20		133.58±6.68	12.78±1.38	23.93±1.67

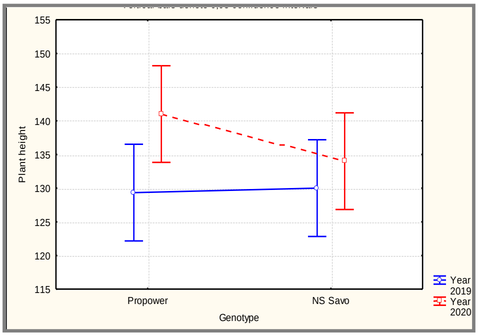
Parameter	**Genotype**		**Year**		**G x Y**	
LSD	**0.5**	**0.1**	**0.5**	**0.1**	**0.5**	**0.1**
Plant height	7.180^{ns}	10.446^{ns}	7.180*	10.446*	10.154*	14.773*
Spike length	1.159*	1.686*	1.159^{ns}	1.686^{ns}	1.639*	2.384*
Green biomass yield	0.519*	0.756*	0.519^{ns}	0.756^{ns}	0.735*	0.340*

Table 4. ANOVA for rye plant height.

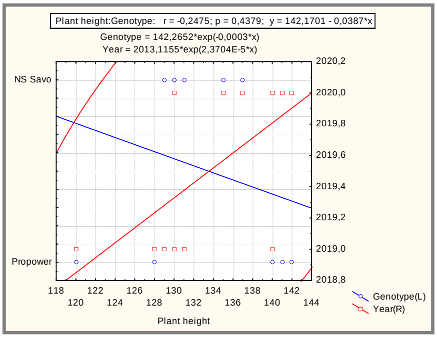
Effect	**SS**	**Degr. of Fr.**	**MS**	**F**	**p**
Intercept	214134.0	1	214134.0	7362.770**	0.000
Genotype	30.1	1	30.1	1.034^{ns}	0.339
Year	184.1	1	184.1	6.330**	0.036
G x Y	44.1	1	44.1	1.516^{ns}	0.253
Error	232.7	8	29.1		

** significant at 0.01; ^{ns}- not significant.

The interaction of the studied factors (G × Y) showed no significant affect in rye plants height (p>0.05). Rye (*Secale cereale* L.) is an ideal crop for agricultural grain production in regions with less fertile and sandy soils, while post-harvest residues (biomass) can be successfully used for biogas production. The amount of precipitation in the vegetation season 2019 was less compared to the 2020 year of the study as well as in relation to the multiannual average. The amount of precipitation was significantly below the average, which represented poor conditions for the growth and development of plants in all pheno-phases (Table 3). Variations in the temperature and the amount of precipitation during vegetation period rye are the most important factors of the yield instability.



(a)



(b)

Figure 1. (a) Interaction year × genotype for plant height of rye genotypes; (b) Interaction genotype × year for plant height of rye genotypes, 2018/19-2019/20.

In the Serbia, high temperatures and the water deficiency during June resulted in grain yield decrease in many crops (Cakić and Stamenković, 2009; Hübner *et al.*, 2011). The yields are strongly modified by the environment of different temperatures and weather conditions. The drought has become a main limiting factor of the world plant production (Hübner *et al.*, 2011; Glamočlija *et al.*, 2015; Rajičić *et al.*, 2020; Dražić *et al.*, 2021). The present results confirm the opinion of many authors that the traits analyzed are genetically determined but are strongly modified by the environment and weather conditions (Popović *et al.*, 2020a).

The plant height was significantly influenced by the year and the G × Y interaction (Table 1). Plant height has positive correlation with yield indicating that taller rye plants have higher yield (Table 8). Plant height were positively correlated with grain yield in the dry environments.

Plant height is one of the critical traits affected by drought in cereals. Low moisture reduces photosynthesis and metabolite/nutrient translocation in wheat, especially during the stem elongation stage, resulting in reduced height (Nsair *et al.*, 2020). The results of the field experiments indicated that there was variation for grain yield under drought stress among genotypes. The introduction of breeding programs for stress conditions is likely to increase in view of the predicted increase in the occurrence of high temperatures and droughts (Spyridonidis *et al.*, 2020; Milanović *et al.*, 2020).

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. 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. Cereals as energy sources in the function of circular economy solution for the use of cereal straw should be the subject of further research. 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 (Dražić *et al.*, 2021).

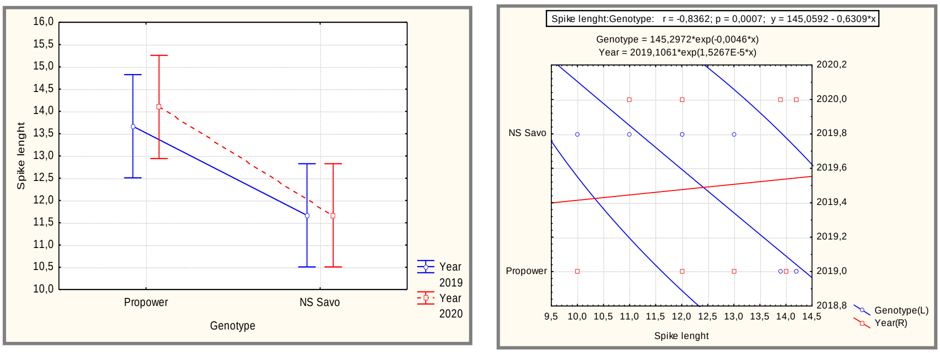
Spikes length of rye genotypes

Genotype had a statistically significant effect on spike length in plants of studied rye genotypes, $p<0.05$. There were no statistically significant differences between the studied years and interactions of year × genotype for the tested parameter, $p>0.05$, Tables 3, 5. The mean of spike lengths in genotypes G1 and G2 was about 13.88 and 11.66 cm, respectively. The more favourable year for the tested parameters was 2019/2020 (12.88 cm) compared to 2018/2019 (12.67 cm), Tables 3, 5.

Table 5. ANOVA for spike length, 2018/2019-2019/2020.

Effect	SS	Degr. of Fr.	MS	F	p
Intercept	1958.4	1	1958.40	2585.35**	0.000
Genotype	14.74	1	14.74	19.46**	0.000
Year	0.14	1	0.14	0.19^{ns}	0.670
G x Y	0.14	1	0.14	0.19^{ns}	0.670
Error	6.06	8	0.76		

** indicate significance different at 0.01; ^{ns}- not significant.



(a) (b)

Figure 2. (a) Interaction year × genotype for spike length; (b) Interaction genotype × year for spike length, 2018/2019-2019/2020.

In 2019/2020, genotype G1 (14.10 cm) had a statistically significantly higher tested parameter in relation to the G2 genotype in 2018/2019 (11.66 cm) and 2019/2020 (11.67 cm), and in comparison to the G1 in 2018/2019 (13.66 cm) (Tables 3, Fig. 2a, 2b).

Based on the analysis of variance, it can be concluded that there are highly significant differences in the rye length of spikes between tested genotypes

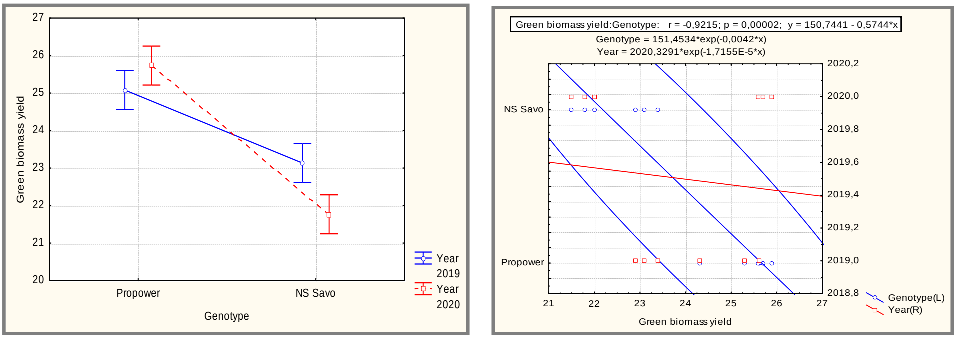
(F_{exp}=19.46**) and no significant differences at studied years (Table 3). There was no significant year × genotype interaction for length of spikes in studied genotypes (p>0.05). The present results confirm the opinion of many authors that the traits analyzed are genetically determined but are strongly modified by environment, of different temperatures and weather conditions (Popović *et al.*, 2020a). Interactions year × genotype had a statistically significant effect on spike length studied rye genotypes (Table 3).

Yield of green biomass

Genotype and genotype × year interaction had a statistically significant effect on green biomass yield, p<0.05 and p<0.01 and no statistically significant differences between the studied years, p >0.05.

Table 6. ANOVA for green biomass yield

Effect	SS	Degr. of Fr.	MS	F	p
Intercept	6870.78	1	6870.78	45143.10	0.0000
Genotype	26.23	1	26.23	172.31**	0.0000
Year	0.38	1	0.38	2.51^{ns}	0.152
G x Y	3.060	1	3.060	20.11*	0.002
Error	1.218	8	0.152		
* and ** indicate significance different at 0.05 and 0.01; ns: not significant.					



(a) (b)

Figure 3. (a) Interaction year × genotype for green biomass yield; (b) Interaction genotype × year for green biomass yield, 2018/2019-2019/2020.

The G1 genotype had, on average, for both studied years a statistically significantly higher yield of green biomass (25.73 t ha^{-1}) compared to the G2 genotype (23.75 t ha^{-1}). The most favourable year for the studied parameter was 2018/2019 (24.11 t ha^{-1}) compared to 2019/2020 (23.75 tha^{-1}), Tables 3, 6.

Genotype G1 had a statistically significantly higher studied parameter in 2019/2020 (25.73 tha^{-1}) compared to genotype G2 in 2019/2020 (21.77 tha^{-1}) and 2018/2019 (23.13 tha^{-1}) and in relation to the genotype G1 in 2018/2019 (25.08 t ha^{-1}) (Tables 3, 6; Fig. 3). Based on the analysis of variance, it can be concluded that there are highly significant differences in the rye green biomass yield between tested genotypes (F_{exp}=172.31**). The interaction of the studied factors (G×Y) exhibits was significant affect in plants biomass yield (F_{exp}=20.11*), Tab. 6. Biomass is a renewable energy source derived from all plants and materials. Genotype and year × genotype interaction had a statistically significant effect on green biomass yield of tested rye genotypes, Table 3.

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_{exp}=937.75**) and years (F_{exp}=28.07**) of investigation (Dražić *et al.*, 2021). Genotype, year and interaction of tested factors (G×Y) had a statistically significant effect on biogas yield. A selection for maximum dry biomass yield in rye breeding should indirectly improve also biogas and methane yield.

Biogas yield

Genotype and genotype × year interaction had a statistically significant effect on biogas yield of tested rye genotypes, $p<0.05$. The G1 genotype had for both studied years a statistically significantly higher biogas yield (260.57 m^3 t^{-1}) on average compared to the G2 genotype (214.58 m^3 t^{-1}). The more favourable year for the studied parameter was 2018/2019 (237.85 m^3 t^{-1}) compared to 2019/2020 (237.30 m^3 t^{-1}), but the difference was not significant (Tables 7, 8).

Table 7. Productivity parameters of rye, Serbia, 2018/2019-2019/2020.

Parameter	Genotype	Biogas yield m^3 t^{-1} fm	Methane yield Nm^{3}ha^{-1} dm	Methane content, %
2018/2019	G1	260.03±0.15	245.03±2.82	56.13±0.11
2019/2020		261.10±0.75	258.13±10.03	56.43±0.55
Average		260.56±0.76	251.58±9.74	56.28±0.39
2018/2019	G2	215.66±0.41	231.00±4.70	52.93±1.19
2019/2020		213.50±1.30	228.03±1.71	52.60±0.70
Average		214.58±1.46	229.52±3.56	52.77±0.89
Average 2018/19		237.85±24.30	238.02±8.43	54.53±1.90
Average 2019/20		237.30±26.08	243.08±17.69	54.52±2.17
Average 2018/19-2019/20		237.58±24.03	240.55±13.48	54.53±1.95

*fm - fresh biomass; dm - dry biomass

Parameter	Genotype		Year		G x Y	
LSD	0.5	0.1	0.5	0.1	0.5	0.1
Biogas yield	1.043*	1.518*	1.043^{ns}	1.518^{ns}	1.475*	2.147^{ns}
Methane yield	7.701*	11.205*	7.701^{ns}	11.205^{ns}	10.891*	15.845*
Methane content	0.994*	1.446*	0.994^{ns}	1.446^{ns}	2.045*	2.045^{ns}

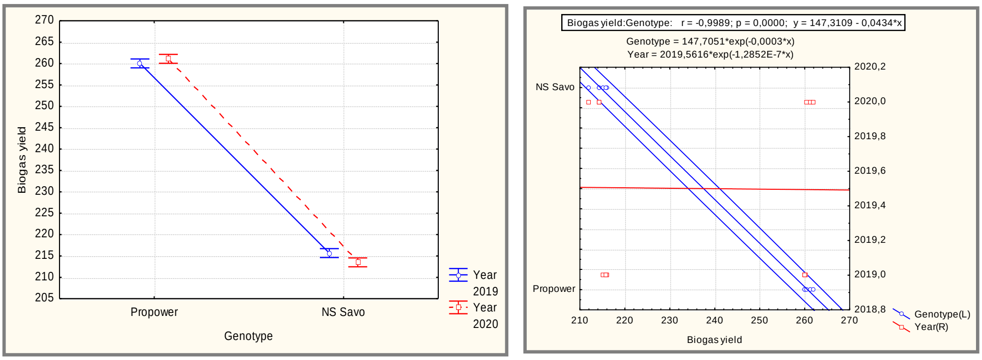
Based on the analysis of variance, it can be concluded that there are highly significant differences in the rye biogas yield between tested genotypes (F_{exp} =

10328.10**) and no significant differences in tested parameter at studied years. The interaction of the studied factors (G × Y) exhibits was significant high affect in biogas yield (F_{exp}=13.40*). Genotype G1 had a statistically significantly higher analyzed parameter in 2019/2020 (261.10 m^3t^{-1}) compared to genotype G2 in 2019/2020 (213.50 m^3t^{-1}) and 2018/2019 (216.66 m^3t^{-1}) (Fig. 4a, 4b).

Table 8. ANOVA for biogas yield.

Effect	SS	Degr. of Fr.	MS	F	p
Intercept	677302.10	1	677302.10	110279.10**	0.0000
Genotype	6343.40	1	6343.40	10328.10**	0.0000
Year	0.90	1	0.90	1.01^{ns}	0.2588
G x Y	7.80	1	7.80	13.40*	0.0072
Error	4.90	8	0.60		

* and ** indicate significance different at 0.05 and 0.01; ns: not significant.



(a) (b)

Figure 4. (a) Interaction year × genotype for biogas yield; (b) Interaction genotype × year for biogas yield, Serbia, 2018/2019-2019/2020.

Genotype and year x genotype interaction had a statistically significant effect on biogas yield of studied rye genotypes, Table 7. Genotype G1 had a statistically significantly higher analyzed parameter in 2020 compared to genotype G2, Fig. 4a. 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_{exp}=3902.25**) and investigated years (F_{exp}=5.32*). The interaction of the investigated factors (G × Y) exhibits was no significant affect in yield (Dražić *et al.*, 2021).

Methane yield

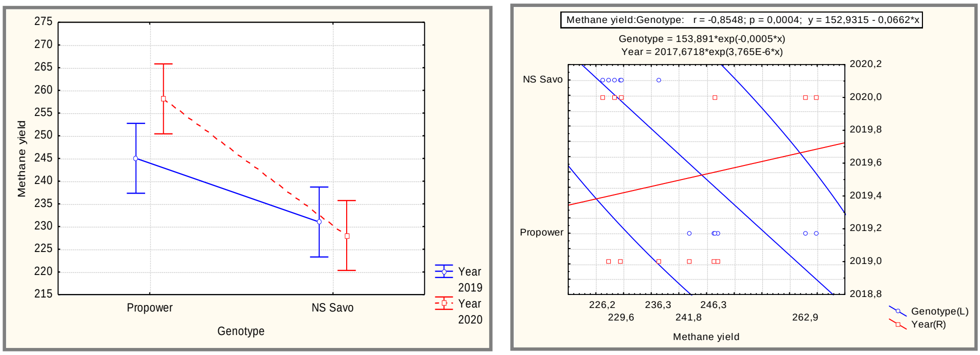
Based on the analysis of variance, it can be concluded that there are highly significant differences in the rye methane yield between tested genotypes (F_{exp}=43.66**) and no significant differences at studied years. The interaction of the studied factors (G×Y) exhibits was significant affect in methane yield (F_{exp}=5.79*), Table 9.

Table 9. ANOVA for methane yield.

Effect	SS	Degr. of Fr.	MS	F	p
Intercept	694371.50	1	694371.50	20752.20	0.0000
Genotype	1460.8	1	1460.8	43.66**	0.0001
Year	77.00	1	77.00	2.30^{ns}	0.16771
G x Y	193.60	1	193.60	5.79*	0.0428
Error	267.70	8	33.50		

* and ** indicate significance different at 0.05 and 0.01; ns: not significant.

The G1 genotype had on average for both studied years a statistically significantly higher methane yield (251.58 m^3ha^{-1}) compared to the G2 genotype (229.52 m^3ha^{-1}).



(a) (b)

Figure 5. (a) Interaction year × genotype for methane yield; (b) Interaction genotype × year for methane yield, 2018/2019-2019/2020.

The more favourable year for the analyzed parameter was 2019/2020 (243.08 m^3ha^{-1}) compared to 2018/2019 (238.01 m^3ha^{-1}), the difference between the years was not significant. In 2019/2020, the genotype G1 had a statistically significantly higher (258.13 m^3ha^{-1}) tested parameter compared to the genotype G2 in 2019/2020 (228.03 m^3ha^{-1}) and 2018/2019 (231.00 m^3ha^{-1}) and genotype G1 in 2018/2019 (245.03 m^3ha^{-1}) (Tables 7, 9; Figures 5a, 5b).

Genotypes and G × Y interaction had a substantial influence on the expression of rye methane yield, Tables 7 and 9. Maximum methane yield per hectare is the main aim of the farmer. Significant ($p < 0.05$) genotypic variation was found for dry matter yield, specific gas yield and methane yield among the 25 genotypes. Ranges were achieved for dry matter yield (0% water content) and methane yield amounting to 2.9 t ha^{-1} and 840 m^3 ha^{-1} respectively, combined with moderate to high heritabilities (0.71–0.98) (Nsair *et al.*, 2020). Anaerobic digestion is an established technology, used to treat a wide variety of organic wastes. It is one of several biological processes that deliver economic and environmental benefits (i.e., producing bioenergy and/or biochemical while treating the organic fraction of waste) (Nsair *et al.*, 2020; Dražić *et al.*, 2021).

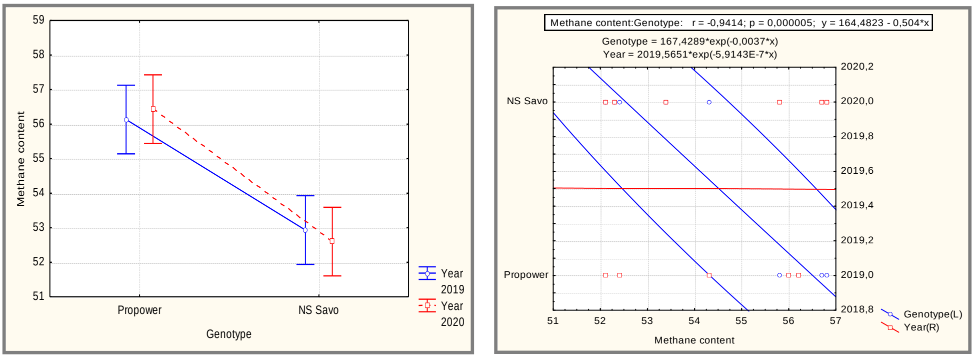
Methane content

Based on the analysis of variance, it can be concluded that there are highly significant differences in the methane content between tested genotypes (F_{exp}=66.55**) and no significant differences during studied years and the interaction of the studied factors (G × Y), Table 10. Genotype had a statistically significant effect on the methane content of the studied rye genotypes, p<0.05. The G1 genotype had on average significantly higher methane content (56.28 %) for both studied years compared to the G2 genotype (52.77 %).

Table 10. ANOVA for methane content.

Parameter	SS	Degr. of Fr.	MS	F	p
Intercept	35675.71	1	35675.71	63992.30	0.0000
Genotype	37.10	1	37.10	66.55**	0.0000
Year	0.00	1	0.00	0.01^{ns}	0.16771
G x Y	0.30	1	0.30	0.54^{ns}	0.0428
Error	4.46	8	0.56		

* and ** indicate significance different at 0.05 and 0.01; ns: not significant.



(a) (b)

Figure 6. (a) Interaction Y × G for methane content (b) Interaction G × Y for methane content, 2018/2019-2019/2020.

The more favourable year for the tested parameter was 2018/2019 (54.53%) compared to 2019/2020, where the difference between the years was not significant. In 2019/2020, the genotype G1 had (54.53%) statistically significantly higher tested parameter compared to the genotype G2 in 2019/2020 (52.60%) and 2018/2019 (52.93%), Tables 7, 10.

Application of varietal production technology improved increasing the yields (Popović *et al.*, 2020b). The biogas production technology has improved over the last years for the aim of reducing the costs of the process, increasing the biogas yields, and minimizing the greenhouse gas emissions (Spyridonidis *et al.*,2020).

Correlations of tested traits.

Correlations of the tested traits of rye varieties are shown in Table 11. A positive statistically very significant correlation was achieved between green biomass yield and spike length (r=0.82**), green biomass yield and biogas yield (r=0.93**), green biomass yield and methane content (r=0.90**).

Table 11. Correlation of tested traits of rye genotypes, 2018/2019-2019/2020.

Variable	Plant height	Spike length	Green biomass yield	Biogas yield	Methane yield	Methane content
PH	1.00	0.30^{ns}	0.26^{ns}	0.25^{ns}	0.43*	0.22^{ns}
SL	0.30^{ns}	1.00	0.82**	0.83**	0.84**	0.88**
GBY	0.26^{ns}	0.82**	1.00	0.93**	0.87**	0.90**
BY	0.25^{ns}	0.83**	0.93**	1.00	0.86**	0.94**
MY	0.43^{*}	0.84**	0.87**	0.86**	1.00	0.85**
MC	0.22^{ns}	0.88**	0.90**	0.94**	0.85**	1.00

^{ns}- not significant; * and ** indicate significance different at 0.05 and 0.01; PH-Plant height; SL-Spike length; GBY-Green biomass yield; BY-Biogas yield; MY-Methane yield; MC-Methane content.

A positive statistically significant correlations were achieved between the methane yield and plant height (r=0.43*), Table 11.

A positive and statistically high significant correlations were achieved between spike length and biogas yield (r=0.83**), and spike length and methane yield (r=0.84**), as well as the spike length and methane content (r =0.88**). A positive and statistically high significant correlations were achieved between the methane content and biogas yield (r=0.94**), the methane content and methane yield (r=0.85**), Table 11.

The study results indicate that GBY - green biomass yield in all vegetation seasons was positively and highly significantly correlated with SL-spike lenght, BY-biogas yield, MY- methane yield and MC-methane content, Table 11.

The biomass yield (BY) was in positive very significant dependence on the spike lenght (r =0.83**) and BY was in positive very significant dependence with the methane yield (r =0.86**). Commercial production should be economically and environmentally friendly so that renewable fuels could be an adequate replacement for fossil fuels (Milanović *et al.*, 2020; Popović *et al.*,2020a). The correlative dependence of the GY- grain yield in the vegetation seasons was positive and highly significant with BY-biogas yield as established by Popović *et al.,* (2020a) and Rakaščan *et al.,* (2021).

The environment can have a crucial influence on plants production in particular regions, even more so when production environments are different from optimum breeding environments (Janković et al., 2016; Popović *et al.,* 2020b; Tmušić *et al.,* 2021). Positive highly significant correlation was obtained between grain yield and biogas yield (r=0.98). Then, positive significant correlation were between grain yield and plant height (r=0.76) and grain yield and precipitation (Popović *et al.,* 2020a).

Plant height were positively correlated with grain yield in the dry environments (Mackay *et al.,* 2009; Sarto *et al.,* 2017; Božović *et al.,* 2020; Mihailovic *et al.,* 2020; Siekmann *et al.,* 2021). The results of the field experiments indicated that there was variation for grain yield under drought stress among genotypes. The introduction of breeding programs for stress conditions is likely to increase in view of the predicted increase in the occurrence of high temperatures and droughts (Sahebi *et al.,* 2001; Manoj *et al.,* 2014; Lakew *et al.,*2021).

The means value of length of rye spikes were significantly correlated for all analyzed traits (Table 11). The majority of the studied traits were correlated to each other. The strongest correlations were observed between GBY and BGY, GBY and MC, BGY and MC, GBY and MY, as well as between MY and MC. Markedly weaker correlations were noticed between PH and the majority of the other traits, Table 11. Grain quality, as well as agronomic important traits controlling plant height, heading date, thousand-grain weight, or yield, reveal a continuous phenotypic variation and are genetically controlled by a network of multiple and interacting loci (Popović *et al.,* 2021; Janković *et al.,* 2016). Analysis of variance (ANOVA) revealed that genotypic effects were statistically significant ($p < 0.05$) for all traits.

Achieving high rye grain yield and quality demands a proper choice of the genotype and by applying the optimal production technology. Genotypes of the new generation exhibit a high degree of tolerance against temperature stress. Two genotypes were selected as the object of research in this study: G1 - Propower and G2 - NS Savo. High grain yield of genotype NS Savo in years with different environmental conditions of over 8.84 t ha^{-1}, excellent tolerance to low temperatures, resistance to the most important diseases and lying down, allows this cultivation varieties and in less favorable conditions and achieving very high yields (Đurić *et al.,* 2021).

Rye together with wheat is the most important bread grain. Rye bread stays fresh for a long time, it is rich in vitamins A, B and E, and since it is great digestibility is recommended for the diet of diabetics. Rye grain is the raw material for production starch and the production of spirits. Rye bread and bakery products have an increasing role in a healthy diet as well convalescent diets and people with elevated blood pressure. Rye is a good bread and bioenergy crop.

CONCLUSIONS

Thanks to the development of new technologies for processing bio-waste into energy sources, the growth rate of the use of alternative fuels is growing significantly. Rye is an excellent raw material for the production of healthy-safe food, but also for the production of biofuels. Our study shows that there are genotypic differences among rye for the biogas production and rye biomass yield. Genotype and genotype x year interaction had a statistically significant effect on yield of biogas and methane and methane content in the studied rye genotypes. Genotype G1 had on average for both studied years a statistically significantly higher biogas yield compared to genotype G2. Genotype G1 was selected as a bioenergy crop and is more suitable for biofuel production. A positive statistically very significant correlation was achieved between green biomass yield and spike length (r=0.82**), green biomass yield and biogas yield (r=0.93**), green biomass yield and methane content (r=0.90**).

The review concludes that there is a need for comprehensive high throughput phenotyping of physio-morphological traits that is growth stage-based to improve the efficiency of breeding highly quality drought-tolerant rye but also for the production of highly productive genotypes for biofuel. Although there are achievements, challenges in rye production remain. A selection rye genotypes with maximum dry biomass yield in rye breeding should indirectly improve also biogas and methane yield. Breeding of varieties with high yield potential biomass is desirable for biogas production, and high grain yield potential with high grain qualities is necessary to further advance rye do to high-performance crop with different types of end-use.

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REFERENCES

Amzeri, A., Daryono, B.S., Syafii, M. (2020): Genotype by environment and stability analyses of dryland maize hybrids. *SABRAO J. Breed. Genet.*, 52: 355–368.

Belitz, H.D., Grosch, W. , Schieberle P. (2009): Cereals and cereal products. In: *Food chemistry*, 4th ed., Belitz H.D., W. Grosch, P. Schieberleeds, Springer, Berlin, pp. 670-675.

Bogdanović, D., Ubavić, M. (1995): Agrochemistry. *Institute of Field and Vegetable Crops,* Novi Sad. Serbia.

Božović, D., Popović, V., Rajičić, V., Kostić, M., Filipović, V., Kolarić, Lj., Ugrenović, V., Spalević, V. (2020): Stability of the expression of the maize productivity parameters by AMMI models and GGE-biplot analysis. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca.* , 48(3): 1387-1397. doi: 10.15835/nbha48312058

Cakić, M., Stamenković O. (2009): Sustainable Technologies biofuels. Tempus 158989 – *JPHES,* 1-59.

Collins, S.R.A., Wellner, N.,.Martinez Bordonado, I., Harper, A.L., Miller, C.N., Bancroft, I., Waldron K.W. (2014): Variation in the chemical composition of wheat straw: the role of tissue ratio and composition. *Biotechnology for Biofuels, 7,* 121.

Cvijanović, V., Cvijanović, G., Rajičić, V., Marinković, J., Đukić, V., Bajagić, M., Đurić, N. (2022): Influence of different methods of application of effective microorganisms in nutrition of wheat on weight by 1000 grains, yield, and content of crude wheat proteins (Triticum sp). *Cereal Research Communications,* 1-10. https://doi.org/10.1007/s42976-021-00226-1

Dražić, N., Rakaščan, N., Radojević, V., Popović, D., Ikanović, J., Popovic, V., Petkovic, Z. (2021): Cereals as energy sources in the function of circular economy. *Agriculture and Forestry, 67* (3):159-169.

Djuric, N., Prodanovic, S., Brankovic, G., Djekic, V., Cvijanovic, G., Zilic, S., Dragicevic, V., Zecevic, V., Dozet, G. (2018): Correlation-Regression Analysis of Morphological-Production Traits of Wheat Varieties. *Romanian Biotechnological Letters*, *23*(2): 13457-13465. doi: 10.26327/RBL2017.71

Đekić, V., Milivojević, J., Staletić, M., Đurić, N., Terzić, D. (2017): Influence of the year on grain yield and quality of Kragujevac rye variety. *Journal of Institute of PKB Agroekonomik, 23*(1-2): 113-118.

Đekić, V., Milivojević, J., Branković, S. (2018): The interaction of genotype and environment on yield and quality components in triticale. *Biologica Nyssana*, *9*(1): 45-53. doi: 10.5281/zenodo.1470850

Đurić, N., Poštić, D., Cvijanović, V., Branković, G., Đekić, V., Cvijanović, G. (2021): PKB Zmaj – a new variety of winter rye created at Institute of PKB Agroekonomik. *Selekcija i semenarstvo,* 27(1):17-23.

Egea-Gilabert, C., Pagnotta, M.A., Tripodi, P. (2021): Genotype × Environment Interactions in Crop Breeding. *Agronomy 11*, 1644. https://doi.org/10.3390/agronomy11081644

Egea-Gilabert, C., Pagnotta, M.A., Tripodi, P. (2021): Genotype × Environment Interactions in Crop Breeding. *Agronomy, 11*, 1644. https://doi.org/10.3390/agronomy11081644

FAO (2019): FAOSTAT, Available at: www.fao.org.

Glamočlija, Đ., Janković, S., Popović, V., Filipović, V., Kuzevski, J., Ugrenović, V. (2015): Alternative crop species in conventional and organic cultivation system. In Serbian. *Monograph.* Belgrade, Serbia. ISBN: 978-86-81689-32-5. P.1-350.

Holliger, Ch., Fruteau de Laclos, H., Hack G. (2017): Methane Production of Full-Scale Anaerobic Digestion Plants Calculated from Substrate's Biomethane Potentials Compares Well with the One Measured On-Site. *Front. Energy Res.*, | https://doi.org/10.3389/fenrg.2017.00012

Hübner, M., Oechsner, H., Koch, S., Seggl, A., Hrenn, H., Schmiedchen, B., Wild, P., Miedaner, T. (2011): Impact of genotype, harvest time and chemical composition on the methane yield of winter rye for biogas production. *Biomass and Bioenergy. 35*(10): 4316-4323. https://doi.org/10.1016/j.biombioe.2011.07.021

Ikanovic, J., Popovic, V., Trkulja, V., Zivanovic, Lj., Lakic, Z., Pavlovic, S. (2013): Morphological characteristics of the interspecies hybrid between sorghum and sudan grass under intensive nitrogen nutration. *Genetika*, *45*(1): 31-40.

Ikanović, J., Živanović, LJ., Popović, V., Kolarić, Lj., Dražić, G., Janković, S., Čurović, M., Pavlović, S. (2018): The possibility of increased use of maize as a bioenergetic. *Journal of Institute of PKB Agroekonomik*, 24(1-2): 49-58.

Jankovic, S., Popovic, V., Ikanovic, J., Rakic, S., Kuzevski, J., Gavrilovic, M. (2016): Productivity traits of rye (*Secale cereale*), Khorasan wheat (*Triticum turgidum, ssp*. Taranicum MCKEY) and quinoa (*Chenopodium quinoa* Willd) grown on degraded soil. *Romanian Agricultural Research, 33*: 283-290. DII 2067-5720RAR2016-148.

Janković, S., Glamočlija, Đ., Ikanović, J., Rakić, S. (2019): Secondary crop products. *Monograph*, Belgrade. SBN 978-86-81689-41-7. 20-70.

Jordanovska, S., Jovovic, Z., Dolijanovic, Z., Dragicevic, V., Brankovic, G., Djekic, V. (2018): Nutritional Properties of Macedonian Landraces of Small Grain Cereals as a Source of New Genetic Variability. *Genetika*, Belgrade, 50(3): 863-883.

Kartina, N., Purwoko, B.S., Dewi, I.S., Wirnas, D., Sugiyanta, A. (2019): Genotype by environment interaction and yield stability analysis of doubled haploid lines of upland rice. *SABRAO Journal of Breeding and Genetics*. 51(2:) 191-204.

Khadka, K., Earl, H.J., Raizada, M.N., Navabi, A.A. (2020): Physio-Morphological Trait-Based Approach for Breeding Drought Tolerant Wheat. *Front. Plant Sci.* 11:715. doi: 10.3389/fpls.2020.00715

Kota, S., Singh, S. S., Mohapatra, T., Singh, A. M., Bhadana, V. P., Ravichandran, S. (2013): Genotype× environment interaction analysis for grain yield in new plant type (NPT) wheat derivatives. *SABRAO Journal of Breeding and Genetics*. 45,3: 382- 390.

Lakew, B., Eglinton, J., Henry, R.J., Baum, M., Grando, S., Ceccarelli, S. (2011): The potential contribution of wild barley (*Hordeum vulgare* ssp. *spontaneum*) germplasm to drought tolerance of cultivated barley (*H. vulgare* ssp. *vulgare*). *Field Crops Res*. *120* (1): 161-168.

Lakić, Ž., Glamočlija, Đ., Kondić, D., Popović, V., Pavlović, S. (2018): Fodder plants and cereals in the function of protecting the land from degradation. *Monograph.* Faculty of Agriculture, Banja Luka. ISBN 978-99938-93-47, pp. 1-405.

Lakić, Ž., Predić, T., Đurđić, I, Popović, V. (2020): Recultivation of degraded soil due to mining activity without adding organic layers of soil using Alfalfa and mixtures of grass legumes. Agriculture and Forestry, 66(4): 223-237.

Luković, K., Prodanović, S., Perišić, V., Milovanović, M., Perišić, V., Rajičić, V., Zečević, V. (2020): Multivariate analysis of morphological traits and the most important productive traits of wheat in extreme rainfall conditions. *Applied Ecology and Environmental Research, 18*(4): 5857-5871. https://dx.doi.org/10.15666/aeer/1804_58575871

Ljubičić, N., Popović, V., Ćirić, V., Kostić, M., Ivošević, B., Popović, D., Pandžić, M., Seddiq, El.M., Janković, S. (2021): Multivariate Interaction Analysis of Winter Wheat Grown in Environment of Limited Soil Conditions. *Plants*. 10(3): 604; https://doi.org/10.3390/plants10030604

Mackay, T.F.C., Stone, E.A., Ayroles, J.F. (2009): The genetics of quantitative traits: challenges and prospects. *Nat. Rev. Genet.* 10: 565–577. doi: 10.1038/nrg2612

Manoj, Kanti D. (2014): Assessment of genotype × environment interactions for grain yield in maize hybrids in rainfed environments. *SABRAO Journal of breeding and Genetics*, 46(2): 284-292.

Mihailović A, Jovanović Lj., Rajičić V. (2020): Climate Change as a Threat to Global Economy, Book of Proceedings, *GEA Conference*, Podgorica, MNE. 1:140-150.

Milanović, T., Popović, V., Vučković, S., Popović, S., Rakaščan, N., Petković, Z. (2020): Analysis of soybean production and biogas yield to improve eco-marketing and circular economy. *Economics of Agriculture*, Belgrade, 67(1):141-156.

Nsair, A.; Onen Cinar, S.; Alassali, A.; Abu Qdais, H.; Kuchta, K. (2020): Operational Parameters of Biogas Plants: A Review and Evaluation Study. *Energies, 13,* 3761. https://doi.org/10.3390/en13153761

Peng, W., Pivato, A. (2017): Sustainable Management of Digestate from the Organic Fraction of Municipal Solid Waste and Food Waste Under the Concepts of Back to Earth Alternatives and Circular Economy. *Waste Biomass Valorization* 10, 465–481.

Pham C.H., Triolo J.M., Cu T.T.T., Pedersen L., Sommer S.G. (2013). Validation and recommendation of methods to measure biogas production potential of animal manure. Asian-Australas *Journal of Animal Science* 26 (6): 864-873. https://doi.org/10.5713/ajas.2012.12623

Popović V., Vučković S., Jovović Z., Ljubičić N., Kostić M., Rakaščan N., Glamočlija-Mladenović M., Ikanović J. (2020a): Genotype by year interaction effects on soybean morpho-productive traits and biogas production. *Genetika,* Belgrade, 52(3):1055-1073.

Popovic V, Ljubicic N, Kostic M, Radulovic M, Blagojevic D, Ugrenovic V, Popovic D, Ivosevic B (2020b): Genotype × Environment Interaction for Wheat Yield Traits Suitable for Selection in Different Seed Priming Conditions. *Plants.* 9 (12): 1804; https://doi.org/10.3390/plants9121804

Provenzano, M.R., Cavallo, O., Malerba, A.D., Fabbri, C., Zaccone, C. (2018): Unravelling (maize silage) digestate features throughout a full-scale plant: A spectroscopic and thermal approach. *J. Clean. Prod.* 193: 372–378.

Rajičić, V., Popović, V., Perišić, V., Biberđžić, M., Jovović, Z., Gudžić, N., Mihailović, V., Čolić, V., Đurić, N., Terzić D. (2020): Impact of Nitrogen and Phosphorus on Grain Yield in Winter Triticale Grown on Degraded Vertisol. *Agronomy,* 10(6): 757-767. https://doi.org/10.3390/agronomy10060757

Rajičić, V., Terzić, D., Babić, V., Perišić, V., Dugalić, M., Đokić, D., Branković, S. (2021): Yield components and genetic potential of winter wheat on pseudogley soil of western Serbia. *Biologica Nyssana, 12*(2): 55-64. doi: 10.5281/zenodo.5759859

Rakaščan, N., Dražić, G., Živanović, Lj., Ikanović, J., Jovović, Z., Lončar, M., Bojović, R., Popović, V. (2019): Effect of genotypes and locations on wheat yield components. *Agriculture and Forestry,* 65(1):233-242.

Rakaščan, N., Dražić, G., Popović, V., Milovanović, J., Živanović, Lj., Remiković-Aćimić, M., Milanović, T., Ikanović, J. (2021): Effect of digestate from anaerobic digestion on *Sorghum bicolor* L. production and circular economy. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca.* 49(1): 1-13. DOI: 10.15835/nbha12270

Richards, B. K., Herndon, F. G., Jewell, W. J., Cummings, R. J., White, T. E. (1994): In situ methane enrichment in methanogenic energy crop digesters. *Biomass and Bioenergy,* 6(4):275-274.

Sahebi J., Ghahreman, A., Rahiminejad, M.R. (2001): A morphologic and taxonomic study of *Hordeum murinum sensu* lato (Poaceae: *Triticeae*) in Iran. *Pakistan Journal of Botany,* 33(2): 133-141.

Sarto, M. V. M., Sarto, J. R. W., Rampim, L., Bassegio, D., da Costa, P. F., and Inagaki, A. M. (2017). Wheat phenology and yield under drought: a review. *Aust. J. Crop Sci.* 11, 941–946. doi: 10.21475/ajcs17.11.08.pne351

Siekmann, D., Jansen, G., Zaar, A., Kilian, A., Fromme, F.J., Hackauf, B.A. (2021): Genome-Wide Association Study Pinpoints Quantitative Trait Genes for Plant Height, Heading Date, Grain Quality, and Yield in Rye (*Secale cereale* L.). *Front. Plant Sci.* 12:718081. doi: 10.3389/fpls.2021.718081

Terzić, D., Popović V., Malić N., Ikanović, J., Rajičić, V., Popović, S., Lončar, M., Lončarević, V. (2019): Effects of long-term fertilization on yield of siderates and organic matter content of soil in the process of recultivation. *The Journal of Animal Plant Sci.* 29(3):790-795

Tmušić, N., Ćirić, S., Nikolić, K., Knežević, J., Rajičić, V. (2021): Effects of different fertilization treatments on the yield performance, yield parameters and grain quality of winter wheat grown on vertisol soil type. *Applied Ecology and Environmental Research, 19*(6): 4611-4627.

Spyridonidis, A.; Vasiliadou, I.A.; Akratos, C.S.; Stamatelatou, K. (2020): Performance of a Full-Scale Biogas Plant Operation in Greece and Its Impact on the Circular Economy. *Water, 12:* 3074. https://doi.org/10.3390/w12113074

www.kolumbus.fi. Retrieved 2.11.07. Basic Information on Biogas Archived 6 January 2010 at the Wayback Machine, 2007.

Zhu, F. (2018): Triticale: nutritional composition and food uses. *Food Chemistry*, 241: 468–479. https://doi.org/10.1016/j.foodchem.2017.09.009.

Review paper

Bojanić Rašović, M., Savić, S., Stojanov, I. (2022): Significance of European foulbrood of honey bees diagnostics in Montenegro. Agriculture and Forestry, 68 (1): 321-330. doi:10.17707/AgricultForest.68.1.20

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SIGNIFICANCE OF EUROPEAN FOULBROOD OF HONEY BEES DIAGNOSTICS IN MONTENEGRO

SUMMARY

The European foulbrood disease of honey bees is a contagious disease primarily of open and rarely of covered bee brood caused by gram positive round bacteria *Melissococcus plutonius*. From the larvae that died of the European foulbrood disease also other bacteria have been isolated: *Enterococcus faecalis, Achromobacter euridica, Paenibacillus alvei* and *Brevibacillus laterosporus,* but they do not affect the appearance of the disease. These bacteria are involved in the process of degradation of dead larvae. European foulbrood disease is on the list of dangerous infectious diseases of the International Organization of epizootic diseases (OIE). Due to the weakening and deterioration of the affected bee colonies, the disease leads to great losses. At the outbreak of the disease crucial influence have adverse environmental conditions and the mistakes in technology of bee breeding. Symptoms of the disease are visible on the open bee brood; the larvae change color to pale yellow, then dark brown, with semi - solid consistency and then disintegrate. There are no data on the presence of this disease in Montenegro. In order to protect bees from the occurrence European bee brood plague, should be work on the systematic diagnostic and implementation of preventive measures in the apiary. Beekeepers should apply the principles of good beekeeping and good veterinary practice.

Keywords: European foulbrood, *Melissococcus plutonius*, Montenegro, bee disease

INTRODUCTION

European bee brood plague is a contagious disease of primarily uncovered, rarely covered brood caused by gram-positive round bacterium *Melissococcus plutonius (White, 1912), Bailey and Collins, 1983,* fam. *Enterococcaceae*

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(formerly *Streptococcus pluton*) (Figure 1). The worker's and drone's litter are susceptible to the disease, as well as the larvae from which the bee queens develop.

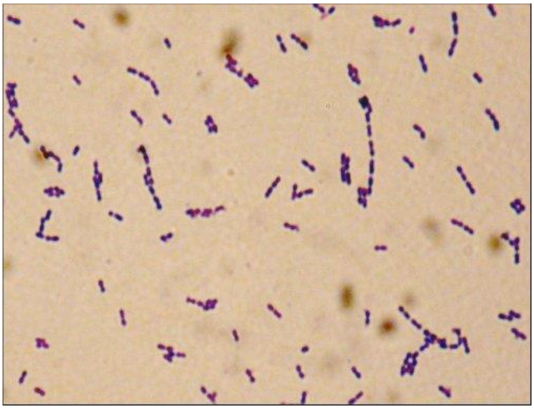

Figure 1. *Melissococcus plutonius,* Gram stain (blue-violet cocci arranged singly, in pairs or short chains) (Lundgren and Johansson, Forsgren et al. 2013)

Bacteria *Enterococcus faecalis, Achromobacter euridice, Paenibacillus alvei* and *Brevibacillus laterosporus* were also isolated from larvae that died from European plague. These bacteria do not affect the occurrence of the disease, but they do affect the process of decomposition of dead larvae (Forsgren, 2010). Although the disease is called the European plague, it is widespread throughout the world and is a growing problem (Mohan Rao et al. 2011). Except in Europe, it is present in North, Central and South America, Asia and Africa. In Europe, it is present in Spain, Switzerland, Sweden, Belgium, Hungary, Great Britain, Italy, Russia, and Belarus. So far, she has not been diagnosed in New Zealand (Bojanić Rašović, 2018c, Anon. 2019, Biová, 2021). It is on the list of dangerous infectious diseases of the International Organization for Epizootics (OIE) and the Rulebook on the classification of infectious animal diseases, the manner of reporting the occurrence or suspicion and reporting of infectious animal diseases (SLCG 92/2017) (Bojanić Rašović, 2018b).

Basic characteristics of European bee brood plague

The occurrence of the disease is crucially influenced by unfavorable environmental conditions and errors in beekeeping technology. Climate change, rainy springs and summers, long winters, long retention of bees in the hive, poor grazing, lack of pollen, lack of nectar, a sharp increase in the amount of nectar, a small number of bees that feed brood, nosemosis and other bee diseases, loss of bee queens, microclimatic conditions in the hive (increased humidity), overheating or chilled litter, errors in technology, bee poisoning, stress, etc. are factors that influence the onset of the disease. Low temperatures and poisoning very often precede the appearance of European bee brood plague.

Due to the weakening and decay of diseased bee colonies, the disease leads to large losses. The disease most often occurs in the spring - when bee colonies reach the largest population, but it can also occur in the autumn (Anon. 2015). The European bee brood plague spreads in the same ways as the American bee brood plague. Healthy bee colonies are usually infected through bees that take food through robberies from diseased colonies; the disease can also be transmitted by beekeepers through infected equipment. Within the hive itself, the disease is transmitted by bees that feed the larvae. The causative agent can also be transmitted through the sting of the *Varroa destructor* mite. The manifestation of clinical signs of the disease occurs only in weak and insufficiently fed colonies with weakened immunity and insufficient number of bees that feed the brood. Regardless of the presence of the causative agent of the disease, the disease will not occur if the colony is strong, if there is enough food, if the litter is warm, etc. Larvae 1-2 days old are most often infected. Infection of larvae in the open litter occurs by ingesting food contaminated with *Melissococcus plutonius*. Bacteria multiply quickly in the middle intestine of bees, because they have good conditions and enough food. Depending on the severity of the infection and the amount of food available, diseased larvae will die or survive. The mortality rate of larvae directly depends on the amount of bacteria ingested, the number of bees that feed brood and the amount of ingested food (Lolin, 1991). Infected young larvae become transparent, lose their pearly white color, and due to the reduction of internal pressure, they stretch out and turn towards the aperture of the cell; due to the extension of the larva, the tracheal system is clearly visible (Fig. 2); larvae change color to pale yellow, then dark brown, semi - liquid, gooey consistency, after which they disintegrate (Figure 3).

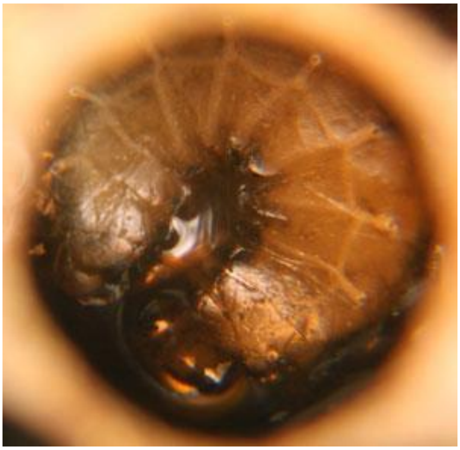

Figure 2. Larvae infected with *Melissococcus plutonius* - well visible tracheal system
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

Figure 3. The dead larva is extended towards the aperture of the cell, it is yellowish to dark brown
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

Larvae usually die at the age of 4-5 days, rarely dying after cell closure. The symptoms of the disease are noticeable on an open litter, unlike the American plague where the symptoms are usually visible on a covered litter. The content of the larvae does not stretch as is characteristic of the American bee plague litter. The stretching test is done by stretching the contents of the dead larvae, usually with the help of a toothpick, a match or a wooden stick (Figure 4).

Figure 4. Slightly stretchable content of larvae killed by European plague
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

If some of the diseased larvae are closed, the appearance of the litter as a whole resembles that of a litter infected with the American plague ("streaked", "scattered" litter). The appearance of a scattered litter is a consequence of death of a certain number of larvae in uncovered and the death or survival of some larvae in covered cells. Holes appear on the lids of cells with diseased or dead larvae - similar to the American plague litter. Holes in the lids appear as a consequence of cleaning the cells and expelling the pathological material by the cleaning bees. Sometimes, the contents of the larva can be weakly extensible - up to 1.5 cm (in the case of American plague, the extensibility is about 2.5 cm) (Bojanić Rašović, 2018a).

Depending on which of the bacteria dominates during the secondary infection, the diseased litter can have a different smell: stale or sour smell, the smell of rotten cheese, rotten fish, the smell of vinegar, but it can often be odorless. Drying of dead larvae creates "scabs" that do not stick to the bottom of the cell and are easily expelled (Figures 5 and 6). (Anon. 2018).

Figure 5. Changes on the larva from the moment of infection to its death, decay and drying in the scab (Ruoff, Forsgren et al. 2013)

Figure 6. Larvae at different stages of development and disease (Forsgren et al. 2013)

The litter observed as a whole is "streaked" - "scattered" (Figure 7). Litter scattering occurs because some larvae die in uncovered cells, and some die or survive in covered cells. Scattered brood also occurs in some other bee diseases.

Figure 7. "Scattered" bee brood infected with European plague
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

At the same time, there are eggs, larvae in different stages and covered cells with dead or healthy dolls on the same honeycomb surface.

Most diseased larvae are detected and removed by cleaning bees. Due to the multiplication of pathogens in the midgut of the larva, infected larvae have a greater need for food. Caregivers bees recognize and expel larvae with excessive food demands. In this way, a strong bee colony can eliminate diseased larvae and keep the European plague under control. Some infected larvae receive enough food to survive and thus prolong the duration of the disease. They pass into the pupal stage, and then into the adult stage. Such puppets and adult forms are stunted - they have a smaller mass compared to the healthy ones and they excrete the pathogen through the feces. Therefore, the disease is most often repeated in treated colonies in the following years, because the causative agent of the disease is constantly present in the hive.

Honeycombs with a diseased litter measuring 10 x 10 cm are taken for laboratory testing. It is best to send honeycombs with freshly dead larvae. Examination of stained microscopic preparations from dead larvae, isolation of the pathogen on nutrient media (Figure 8), as well as molecular diagnostics (PCR) confirm the presence of the pathogen (Ansari et al. 2017).

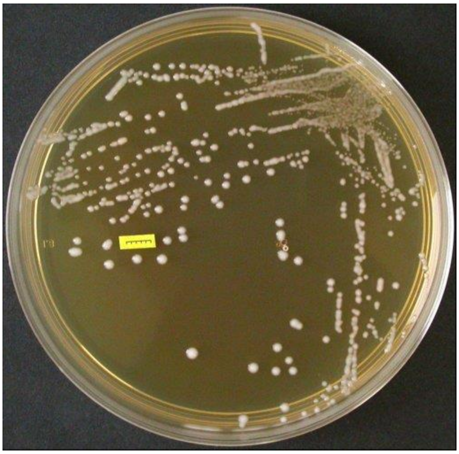

Figure 8. Appearance of *Melissococcus plutonius* colonies on basal medium (Lundgren and Johansson, Forsgren et al. 2013)

For microscopic examination, a diluted aqueous suspension is prepared from the contents of the intestine which is transferred to a microscopic slide and mixed with a 5% aqueous solution of nigrosine (Anon. 2018). The aqueous suspension is evenly distributed on 1-2 cm^2 of the glass surface, lightly dried on a flame and observed under a microscope. The presence of a large number of lanceolate cocci (having the appearance of a lancet or spear), measuring about 0.5 x 1.0 μm, arranged individually, in groups, pairs or short chains, almost certainly indicates that it is a European plague litter (Figure 1). *Melissococcus plutonius* is a gram positive bacterium that does not produce spores (Figure 1). However, even if it does not create spores, it is quite resistant in the external environment, to the action of physical and chemical factors and drying. Thanks to its resistance, it can survive the winter period on the walls of honeycomb cells, in feces and wax residues at the bottom of the hive, and be a source of infection in the following years as well. On nutrient media under anaerobic conditions for 4 days it grows in the form of small transparent colonies with a diameter of 1 mm (Figure 8). It is very pleomorphic in culture, meaning it changes shape; it can also occur in the form of sticks, especially in cultures that are stored for several weeks. The isolated bacteria can be detected by agglutination in a test tube with the help of antiserum obtained by immunizing rabbits.

Measures to combat the European bee brood plague

In order to prevent the appearance of the disease, it is necessary for the bee colonies to warm up in time (especially in the spring), in the absence of pasture to be fed, for the bee queen to be changed every two years, and for the equipment and hives to be disinfected regularly; bee colonies need to be strong and have enough quality food - that is, quality honey. When importing beekeeping equipment, preventive disinfection should be done with a 0.5% solution of sodium hypochlorite, for 20 minutes or with radiation of 15 kilogray (Anon. 2017a).

When the disease occurs, if the colony is very weak, and the cells are blocked by a large number of dead larvae, the bee colony is destroyed - burned, as in the case of the American plague. If the colonies are on average strong and there is not a large percentage of dead larvae and untreated cells, measures are taken to cure the diseased colony; these are measures that improve the conditions of beekeeping (brood warming, feeding the colony) and measures aimed at destroying and removing pathogens from the hive (disinfection of equipment and accessories, moving the colony to a clean and disinfected hive, etc.) (Bojanić Rašović et al., 2019, Bojanić Rašović, 2019, Bojanić Rašović, 2020a, Bojanić Rašović, 2020b, Bojanić Rašović, 2021a, Bojanić Rašović, 2021b, Bojanić Rašović, 2021c, Bojanić Rašović, 2021d, Bojanić Rašović, 2021e, Bojanić Rašović, 2021 f).

It should be borne in mind that the appearance of the disease in the same bee colonies can occur again in the following years, if the conditions for beekeeping worsen again (Plavša and Pavlović, 2017). Antibiotics have previously been used to treat this disease, but the effects of this therapy are unsatisfactory and harmful. Antibiotic therapy does not lead to the cure of the disease, but only to its transition to another - hidden form. Antibiotics prevent the healing of the bee colony in such a way that antibiotics enable the survival of infected larvae; infected larvae should be allowed to die to be removed by nurse bees. This eliminates the source of the infection. Antibiotic therapy has also led to the emergence of resistance of pathogenic microorganisms to antibiotics and the emergence of fungal and other diseases of bees. Antibiotics leave harmful residues in honey. For all these reasons, antibiotics are banned for use in beekeeping.

Measures to prevent the occurrence of European bee brood plague in Montenegro

In Montenegro beekeepers have the obligation to report to the veterinary service any suspicion of the occurrence of both this and other dangerous infectious diseases of bees. Measures to diagnose and control infectious diseases are carried out under the supervision of a veterinarian. According to international, as well as national regulations, the European bee brood plague is classified as a dangerous infectious disease and as such it is mandatory for reporting to the International Organization for Epizootics (OIE) (Anon. 2017b, Anon. 2018). The Directorate for Food Safety, Veterinary and Phytosanitary Affairs of Montenegro is obliged to report confirmed cases to the OIE. In Montenegro, there are no data on diagnostic testing of bee colonies for European bee brood plague, nor this disease has been diagnosed in Montenegro. Considering that the disease has been diagnosed in the countries of the immediate and distant environment, in Montenegro the diagnostics and control of beehives for this disease should be carried out. Considering that laboratory diagnostics of European plague is not performed in Montenegro (no isolation of pathogens is performed), it is necessary to work on the introducing of this microbiological method. Also, in Montenegro there is no defined program of measures to combat this disease, which should include the above measures, as timely warming of bee colonies, providing a sufficient amount of honey for overwintering of bees, supplementing with quality food, replacement of bee queen every other year, regular disinfection of equipment and bee hives, burning of very weak bee colonies suffering from European bee brood plague, etc. In order to prevent the occurrence of infectious diseases of bees, also European bee brood plague**,** beekeepers apply the principles of good beekeeping and good veterinary practice (Grubić, 2018, Rašić, 2018).

CONCLUSIONS

European bee brood plague is a contagious disease of primarily open, less often closed brood caused by gram-positive round bacterium *Melissococcus plutonius*. Due to the weakening and decay of diseased bee colonies, the disease leads to large losses. The occurrence of the disease is crucially influenced by unfavorable environmental conditions and errors in beekeeping technology. If be colonies suffering from the European plague are very weak, colonies are destroyed. If bee colonies are on average strong, measures are taken to cure the diseased societies. In order to protect the bees of the European bee brood plague in Montenegro, should be done on introducing diagnostics and implementation of preventive measures in the apiary. In order to prevent the occurrence of infectious diseases of bees, beekeepers should apply the principles of good beekeeping and good veterinary practice.

REFERENCES

Anon. (2015). European foulbrood (EFB). FAO, Apimondia, IZSLT - Istituto Zooprofilattico Sperimentale del Lazio e della Toscana “Mariano Aleandri”, p 1-4.

Anon. (2017a). OIE - Terrestrial Animal Health Code (2017*)*. Chapter 9.3.- Infection of honey bees with *Melissococcus plutonius (European foulbrood),* p 1-4.

Anon (2017b). Pravilnik o klasifikaciji zaraznih bolesti životinja, načinu prijavljivanja pojave odnosno sumnje i odjavljivanja zaraznih bolesti životinja (SLCG 92/2017).

Anon. (2018). OIE Terrestrial Manual: European Foulbrood in honey bees (Infection of honey bees with *Melissococcus plutonius*). Chapter 3.2.3; 1-8.

Anon. (2019). CABI European Foulbrood. (accessed on 8. August 2021); Available online: https://www.cabi.org/isc/datasheet/109547#tooverview.

Ansari, J. M., Al-Ghamdi, A., Nuru, A., Ahmed, A.M., Ayaad, T.H., Al-Qarni, A., Alattal, Y., Al-Waili N.(2017). Survey and molecular detection of *Melissococcus plutonius*, the causative agent of European Foulbrood in honeybees in Saudi Arabia. Saudi Journal of Biological Sciences, 52:6, 1327-1335.

Biová J., Charrière J.D., Dostálková S., Škrabišová M., Petˇrivalský M., Bzdil J., Danihlík J. (2021), *Melissococcus plutonius* Can Be Effectively and Economically Detected Using Hive Debris and Conventional PCR, Insects, 12, 150, 2-11.

Bojanić Rašović M. (2018a): Američka kuga pčelinjeg legla, Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 210, p 12-15.

Bojanić Rašović M. (2018b): Zdravstvena zaštita pčela u Crnoj Gori, Pčelarstvo, Savez pčelarskih organizacija Crne Gore: 14-15.

Bojanić Rašović M. (2018c). Evropska kuga pčelinjeg legla, Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 210, p 16-18.

Bojanić Rašović M., Davidović V., Joksimović-Todorović M. (2019). Importance and measures of health protection of honey bees in Montenegro, Journal of Hygienic Engineering and Design, vol 29 p 50-54

Bojanić Rašović M. (2019): Virusne bolesti pčela, Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 215, p 14-17

Bojanić Rašović M. (2020a). Mikrobiologija, Univerzitet Crne Gore, Podgorica, p 1-411.

Bojanić Rašović M. (2020b). Značaj primjene dobre pčelarske prakse u proizvodnji meda i drugih pčelinjih proizvoda u Crnoj Gori, Pčelarstvo, Savez pčelarskih organizacija Crne Gore, p 1-28.

Bojanić Rašović M. (2021a). Značaj i metode dezinfekcije u stočarstvu (prvi dio), Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 238, p 22-25.

Bojanić Rašović M.(2021b). Značaj i metode dezinfekcije u stočarstvu (drugi dio), Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 239, p 20-23.

Bojanić Rašović M.(2021c). Značaj i metode dezinfekcije u stočarstvu (treći dio), Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 240, p 14-16.

Bojanić Rašović M.(2021d). Dezinfekcija u pčelarstvu (prvi dio), Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 241, p 20-26.

Bojanić Rašović M. (2021e). Dezinfekcija u pčelarstvu (drugi dio), Pčelarstvo, Savez pčelarskih organizacija Crne Gore, No 242, p 18-24.

Bojanić Rašović, M. (2021 f): The most important methods of disinfection in beekeeping. Agriculture and Forestry, 67 (3): 167-176

Forsgren E. (2010): European foulbrood in honey bees. Journal of Intervertebrate Pathology, 103, S5-S9.

Forsgren, E., Budge, G.E., Charrière J.D., Hornitzky M.A.Z. (2013): Standard methods for European foulbrood research. Journal of Apicultural Research, 52:1, 1-14.

Grubić S.(2018). Dobra veterinarska praksa u pčelarstvu, SPOCG, Danilovgrad, p 1-69.

Lolin M.(1991). Bolesti pčela, Naučna knjiga, Beograd, p 1-118.

Mohan Rao K., Rana**,** B. S. Chakravarty, S. K., Sapna K., Anju S. (2011). Characterization of *Melissococcus pluton* from European honey bee (*Apis mellifera* L.) of North-West Himalayas, International Journal of Science and Nature, 2(3): 632- 638.

Plavša N., Pavlović I. (2017). Bolesti pčela, Univerzitet u Novom sadu, Poljoprivredni fakultet, Novi Sad, p 1-134

Rašić S.(2018). Dobra pčelarska praksa, SPOCG, Danilovgrad, p 1-122

https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/ European Foulbrood: A Bacterial Disease Affecting Honey Bee Brood, August 20, 2019, Bee-Health (19.january 2022).

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