BOOK OF PROCEEDINGS

6.

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BOOK OF PROCEEDINGS

The 6th CONGRESS OF THE SERBIAN GENETIC SOCIETY

The present volume gathers the proceedings of the VI Congress of the Serbian Genetic Society that was held in Vrnjačka Banja from 13-17 October 2019. The Congress addressed various fields of genetics, highlighting the most recent advances as well as emerging challenges. The broad range of conference topics was organized in nine sessions and two workshops.

This collection of selected papers covering different areas, including genetics of microorganisms, plants and animals, population genetics, genotoxicology, human genetics, etc. will undoubtedly be of benefit to researchers and scientists from both academia and industry.

All papers included in the present Book of Proceedings were positively reviewed by two referees. Full texts of the accepted contributions are available in electronic form on the website of SGS (http://www.dgsgenetika.org.rs/). Much appreciation is to the authors of all papers submitted and to all reviewers, for their help in editing the Book of Proceedings.

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BOOK OF PROCEEDINGS

The 6th CONGRESS OF THE SERBIAN GENETIC SOCIETY

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The 6th CONGRESS OF THE SERBIAN GENETIC SOCIETY

INFLUENCE OF TOWN WASTEWATERS AND AGRICULTURAL ACTIVITIES ON WATER GENOTOXICITY DURING DIFFERENT RIVER LEVEL REGIMES

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ABSTRACT

Water, being of critical importance for the survival of life on the planet, requires our permanent care and attention. There is a growing concern about the genotoxicity of complex environmental mixtures in surface waters, due to the risk of genetic damage and cancer, both in aquatic organisms and humans. The present study focuses on exploring the status of water pollution in areas with combined industrial and agricultural activities in order to estimate the magnitude of toxicity and genotoxicity using the *Allium* test. We collected samples from the Sava and Danube rivers, upstream and downstream of Šabac (Sava River) and Smederevo (Danube River). In both rivers sampling was done in periods of low and high-water levels. Levels of toxicity were low in both rivers. However, the presence of organic pollution, observed as a higher mean length of the roots, was seen in all sample groups compared to negative control. The highest values of genotoxicity at locations upstream of both Šabac and Smederevo were obtained in samples collected in a period of high-water level, but only the upstream sample from the Sava River had reached a significant level of genotoxicity. This can be considered as a consequence of intensive agricultural activities. Our results indicate that communal town and industrial wastewaters influence river water quality more significantly during low level regimes, while high water levels increase the risk of exposure to chemicals used in agriculture.

Keywords: *Allium* test, genotoxicity, Sava River, Danube River, town wastewaters, agriculture pollution

INTRODUCTION

Nowadays, when it comes to water, the term clean water crisis is increasingly used. It is becoming gradually more apparent that the amount of healthy drinking water is decreasing in the face of the constant increase of the human population and furthermore due to the increase in water consumption brought by a rise in standards.

Considering how limited the amount of drinking water is and how important the water is per se, it is of paramount importance to know not only what pollutes the water, but also what the consequences for the consumers of that water and their offspring might be (SCHWARZENBACH *et al*., 2010). In the past, because of its vast surface area and volume, people have treated the aquatic environment as a convenient place for the disposal of biological and technical waste. However, it has now become apparent that the deposition of toxicants into aquatic ecosystems has led to those toxicants accumulation in both sediments and upstream food chains (SONSTEGARD, 1977; STATHAM *et al*., 1976).

Rivers and lakes cover approximately only 2.3% of the global land surface area on Earth (LEHNER and DOLL, 2004). Thus, actual water quality management requires a clear understanding of how and why water quality differs across space, both within and between different river catchments. Freshwaters are exposed to multiple persistent and evolving threats (REID *et al*., 2019). RICCIARDI and RASMUSSEN (1999) estimated that in North America, 123 species of freshwater animals are near extinction and that the extinction rate in the aquatic system is five-fold higher than on land reaching 4% per decade. According to HE *et al*., (2019) freshwater megafauna populations declined by 88% from 1970 to 2012. Such a high rate of species extinction in aquatic ecosystems is likely caused by pollution originating from activities occurring in the terrestrial environment as well as from changes in artificially induced river flows. More than one third of rivers in the United States (BERNHARDT *et al*., 2005) and even more than 45% of rivers in China (VOROSMARTY *et al.*, 2010) are judged as polluted.

Water pollutants come from three main sources: human and domestic waste (largely biodegradable organic products), industrial waste (mainly toxic waste that readily accumulates in the food chain) and agricultural chemicals (fertilizers and pesticides) that are used to obtain high yields. Many substances that enter the water and air as by-products of industrial production, agriculture, or fossil fuel combustion are potential mutagens and/or carcinogens. Water pollution problems vary widely across regions of the world. In general, agricultural pollution is the least resolved problem.

Industrial and urban wastewater is generally characterized by the presence of extremely complex mixtures containing numerous inorganic and organic compounds. This complexity makes it very difficult to chemically assess the danger posed by polluted water. In these situations, different variants of the *Allium* test are well suited for assessing the toxicity and potential genotoxicity of different water samples (RANK and NIELSEN, 1993; VUJOŠEVIĆ *et al*., 2001; VUJOŠEVIĆ *et al*., 2008; LEME and MARTIN-MORLAES, 2009; RADIĆ *et al.*, 2010).

It can generally be said that the state of waters in Serbia is insufficiently known (ABORGIBA *et al*., 2016; KOSTIĆ *et al*., 2017). Regular bacteriological and chemical

control gives insufficient data on real pollution level. The study of pollution of the Sava and Danube Rivers in the Belgrade region, as the area of their confluence, showed how different chemical analyses can produce different assessments of the river quality and lead to potential misinterpretation of pollution levels (ANTONIJEVIĆ *et al*., 2014).

The present study was motivated by the need for a preliminary estimation of the water quality of two rivers, Sava and Danube, upstream and downstream of the towns Šabac and Smederevo during different water levels. The toxic and genotoxic potential of the water samples was assessed using *Allium* anaphase-telophase test.

MATERIALS AND METHODS

Two samplings were done independently on the Sava and Danube rivers, upstream and downstream of Šabac (Sava River) and Smederevo (Danube River). Samples of surface water from both rivers were collected upstream (loc 1) and downstream (loc 2) from the towns and analysed using the *Allium* anaphase-telophase test. Differences in the degree of pollution between loc1 and loc 2 of each river were considered to be a contribution of urban wastewater. On the Sava River loc 1 was at 44.792769° 19.691358° and loc 2 at 44.745365° 19.762664° (Figure 1a). On the Danube River loc 1 was at 44.658590°, 20.869184° and loc 2 at 44.694314° 20.959865° (Figure 1b). Distances between loc 1 and loc 2 were approximately 10 km in both cases. Sampling was done in periods of low (October/November 2017) and high-water levels (March/April 2018). According to the Republic Hydrometeorological Service of Serbia (http://www.hidmet.gov.rs/eng/hidrologija/index.php) water levels during the first sampling were -68 cm at Sava River and 466 cm at Danube River, while at the second sampling levels were 529 cm and 618 cm, respectively.

a

Figure 1. Locations upstream (loc 1) and downstream (loc 2) of towns: (a) Šabac - Sava River and (b) Smederevo – Danube River.

Following modified procedure for *Allium* anaphase-telophase test (RANK and NIELSEN, 1993) commercial onion bulbs of *Allium cepa,* weighing between 2-4 grams, were set in groups of 12 for each test sample and control groups, and left to grow for five days. For each sampling period 4 test samples were set up. Two control groups were used. As a positive control, we used a known mutagen methyl methane sulfonate MMS (SIGMA M-4016) at a concentration of 10 μg/l. Negative control was synthetic water. In test groups sample water was replenished once a day. Two onion bulbs with the poorest growth were eliminated from each group on the third day.

To determine general toxicity, the length of roots in 10 bulbs was measured in each sample. For genotoxicity analysis root tips were heated in 2N HCl solution for 12 minutes at 65^{o}C and macerated. Five root tips (1-2 mm in length) from one onion were placed on each slide, stained with 2% orcein and squashed. Slides were coded and examined blind.

The level of toxicity was measured as the mean root length in tested samples in comparison to the negative control. To assess level of genotoxicity in each sample and controls (positive and negative) the following aberrations, in anaphase and telophase, were scored: bridges, fragments, vagrant chromosomes, multipolarity, and C-mitoses. A Two-by-Two contingency χ^2 statistical test was employed to determine the significance of differences between the analysed groups including controls, separately for each river.

RESULTS AND DISCUSSION

Šabac and Smederevo are considered as important regional industrial centers positioned on the banks of the large rivers (Sava and Danube). Thus, these rivers have been used for communal and industrial municipal effluent discharge and for transport related to production.

During this study, the water level on the Sava River differed for 597 cm between the first (low water level) and the second (high water level) sampling, while that difference was 152 cm on the Danube.

Roots grew better (for 4.3-12.6 %) in all analyzed river samples comparing to the negative controls, thus pointing to the presence of organic pollution in both rivers. Differences in the average root growth between loc 1 (upstream) and loc 2 (downstream) of the towns were not significant, indicating that communal and industrial wastewaters from both towns were not toxic.

However, the level of genotoxicity was dependent on the water level. We obtained the highest values of genotoxicity at locations upstream of both Šabac and Smederevo, in samples collected in a period of high water level (Figure 2), but only the sample from loc 1 on Sava River was significantly different ($X_{(1)}^2=29.51$; $p<0.001$) from aberrant cells found in the negative control (6.97%). Both towns, besides being regional industrial centers, are situated in regions of intensive agricultural activities. A large increase in river water levels leads to flooding of coastal agricultural land. As a consequence, chemicals used in crop production reach the water, raising the level of genotoxicity upstream of towns.

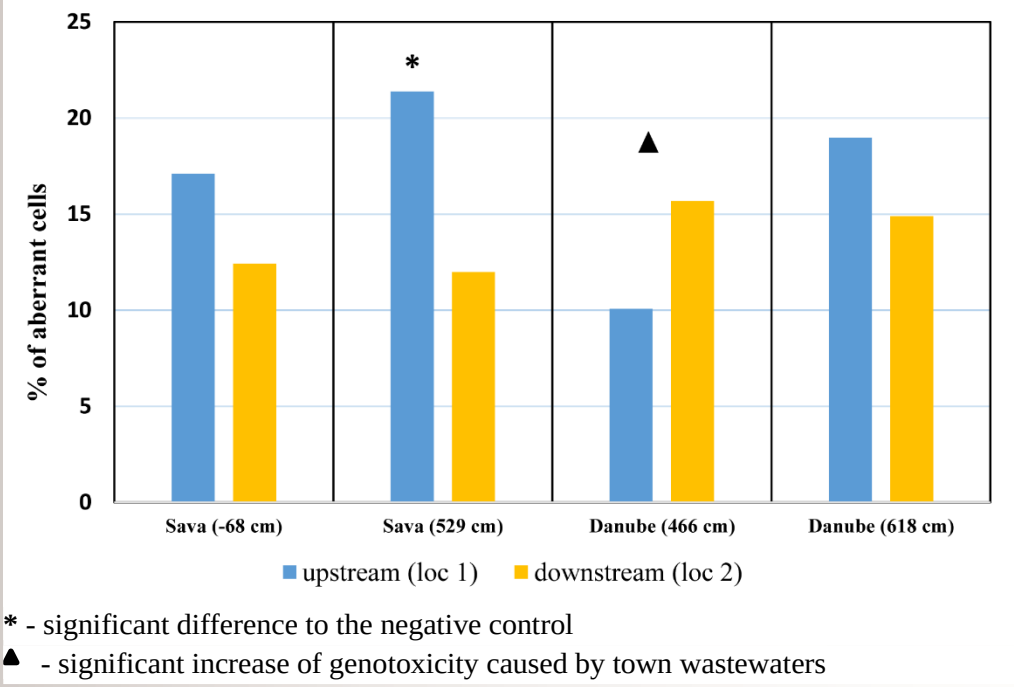

Figure 2. Percentage of aberrant cells in *Allium* anaphase-telophase test for the river water samples collected upstream and downstream of Šabac (Sava River) and Smederevo (Danube River) during low and high water levels (water levels on date of sampling, according to the Republic Hydrometeorological Service of Serbia, are given in brackets).

It is well known that pesticides frequently show genotoxic effects (summarized by LEME and MARTIN-MORALES, 2009). Furthermore, GADEVA and DIMITROV (2008), found that types of chromosomal aberrations are correlated with the concentration of different pesticides, and some of them in concentrations comparable to those used in practice can be designated as potential aneugens. ROSCULETE *et al*. (2018) showed that a higher herbicide concentration increased the percentage of chromosomal aberrations and had a higher mitodepressive effect.

In the period of low water level on the Danube, significant genotoxicity differences were obtained between loc 1 and loc 2 ($X_{(1)}^2$=7.62; p=0.006), indicating that urban wastewaters of Smederevo caused notable pollution. It is important to emphasize that the values at both sites, upstream and downstream, did not differ significantly from the negative control. Similar result was reported by FATIMA and AHMAD (2006); by using the *Allium* test they found increased genotoxicity in the samples of industrial effluence of two cities in India, containing mainly pesticides and heavy metals. Upon applying the same test, GANA *et al*. (2008) obtained increased genotoxicity in the samples from different industrial effluents.

Large rivers have high water flow, which could minimize the effects of chemicals, but local effects could be significant. This means that the potential damage to the environment can be much greater in small rivers. It seems that communal town and industrial wastewaters influence more significantly the river water quality during a low-level regime, while high water levels increase the risk of exposure to chemicals used in agriculture.

CONCLUSION

The increased levels of root growth in all river samples in comparison to negative controls point to the presence of organic pollution in both rivers. Levels of genotoxicity were the highest at points upstream of towns Šabac and Smederevo, in samples collected in periods of high-water levels. This can be a consequence of increased runoff from land characterized by intensive agricultural activities. Levels of river water define which kind of pollution will be the dominant ones. Town communal and industrial wastewaters have a higher impact on river water quality during low level regimes, while high water levels increase the risk of exposure to chemicals used in agriculture.

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REFERENCES

Aborgiba, M., J., Kostić, S., Kolarević, M., Kračun-Kolarević, S., Elbahi, J., Knežević-Vukčević, M., Lenhardt, M., Paunović, Z., Gačić, B., Vuković-Gačić (2016): Flooding modifies the genotoxic effects of pollution on a worm, a mussel and two fish species from the Sava River. Sci. Total Environ., *540*: 358–367.

Antonijević, M.D., M., Arsović, J., Čáslavský, V., Cvetković, P., Dabić, M., Franko, G., Ilić, M., Ivanović, N., *et al.* (2014): Actual contamination of the Danube and Sava Rivers at Belgrade. J. Serb. Chem. Soc., *79*(9): 1169–1184.

Bernhardt, E.S., M.A., Palmer, J.D., Allan, G., Alexander, K., Barnas, S., Brooks, J., Carr, S., Clayton, C., Dahm, J., Follstad-Shah, *et al*. (2005): Synthesizing U.S. river restoration efforts. Science, *308*: 636–637.

Fatima, R.A., M., Ahmad (2006): Genotoxicity of industrial wastewaters obtained from two different pollution sources in northern India: a comparison of three bioassays, Mutat. Res., *609*: 81–91.

Gadeva, P., B., Dimitrov (2008): Genotoxic effects of the pesticides Rubigan, Omite and Rovral in root-meristem cells of *Crepis capillaris* L. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, *65*2(2): 191-197.

Gana, J.M., R., Ordóñez, C., Zampini, M., Hidalgo, S., Meoni, S., M.I., Isla (2008): Industrial effluents and surface waters genotoxicity and mutagenicity evaluation of a river of Tucuman, Argentina, J. Hazard. Mater., *155*: 403-406.

He, F., C., Zarfl, V., Bremerich, *et al*. (2019): The global decline of freshwater megafauna. Glob Change Biol. *25*(11): 3883-3892.

Kostić, J., S., Kolarević, M., Kračun-Kolarević, M., Aborgiba, Z., Gačić, M., Paunović, Ž., Višnjić-Jeftić, B., Rašković, V., Poleksić, M., Lenhardt, B.,Vuković-Gačić (2017): The impact of multiple stressors on the biomarkers response in gills and liver of freshwater breams during different seasons, Sci. Total Environ., *601–602:* 1670–1681.

Lehner, B., P., Doll (2004): Development and validation of a global database of lakes, reservoirs and wetlands. J. Hydr., *296*: 1–22.

Leme, D.M., M.A., Marin-Morales (2009): *Allium cepa* test in environmental monitoring: A review on its application. Mut. Res., *682*: 71–81

Radić, S., D., Stipaničev, V., Vujčić, M., Marijanović Rajčić, S., Širac, B. Pevalek-Kozlina (2010): The evaluation of surface and wastewater genotoxicity using the *Allium cepa* test. Sci. Total Environ., *408:* 1228–1233.

Rank, J-, M.H., Nielsen (1993): A modified *Allium* test as a tool in the screening of the genotoxicity of complex mixtures, Hereditas, *118*: 49-53

Reid, A.J., A.K., Carlson, I.F., Creed, E.J., Eliason, P.A., Gell, P.T.J., Johnson, *et al.* (2019): Emerging threats and persistent conservation challenges for freshwater biodiversity. Biol. Rev., *94*: 849–873.

Ricciardi, A., J.B. Rasmussen (1999): Extinction rates of North American freshwater fauna. Conservation Biology, *13*(5): 1220-1222.

Rosculete, C.A., E., Bonciu, E., Rosculete, L.A., Olaru, (2018): Determination of the environmental pollution potential of some herbicides by the assessment of cytotoxic and genotoxic effects on *Allium cepa*. Int. J. Environ. Res. Public Health, *16*(1), pii: E75. doi: 10.3390/ijerph16010075.

Schwarzenbach, R.P., T., Egli, T.B., Hofstetter, U., von Gunten, B., Wehrli (2010): Global water pollution and human health. Annu. Rev. Env. Resour., *35*: 109–136.

Sonstegard, R.A. (1977): Environmental carcinogenesis studies in fishes of the Great Lakes of North America. Ann. N.Y. Acad. Sci., *298*: 291-269.

Statham, C.N., M.Jr., Melancon, J.J., Lech (1976): Bioconcentracion of xenobiotics in trout bile: A proposed monitoring aid for some waterborne chemicals. Science, *193*: 680-681.

Vorosmarty, C.J., P.B., McIntyre, M.O., Gessner, D., Dudgeon, A., Prusevich, P., Green, S., Glidden, S.E., Bunn, C.A., Sullivan, C.R., Liermann, *et al.* (2010): Global threats to human water security and river biodiversity. Nature, *467*: 555–561.

Vujošević M., S., Anđelković, G., Savić, J., Blagojević (2008): Genotoxicity screening of the river Rasina in Serbia using the *Allium* anaphase-telophase test. Environ. Monit. Assess., *147*(1-3): 75-81.

Vujošević, M., J., Blagojević, V., Vitanović-Martinović, V., Kalafatić (2001): The presence of genotoxic substances in the Danube river five months after the bombardment of industrial zone of Pančevo as revealed by *Allium* anaphase-telophase genotoxicity assay. Acta Vet., *31*(5-6): 373-382.

VARIABILITY OF BEECH (*Fagus sylvatica* L.) POPULATION IN SERBIA BASED ON MORPHOLOGICAL CHARACTERISTICS OF CUPULES

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ABSTRACT

Beech (*Fagus sylavtica* L.) as a species of wide utilization is one of the most important trees in Europe. Taxonomic status of this species is a decades-old problem in the southeastern part of its European distribution. Based on the numerous studies, the analysis of the morphological characteristics of the cupules proved to be informative in terms of selecting certain ecotypes in this area. In order to determine variability between populations we analyzed cupules from 12 natural beech populations in Serbia, from different elevations (300 to 1350 m.a.s.l). Five quantitative morphological parameters were analyzed: cupule length (without the petiole), length of the longest valve, width of the longest valve, petiole length and distance between the base of the longest valve and the petiole ("connecting piece"). The length of the petiole and "connecting piece" were shown to be the most variable characteristics. The analysis of variance showed that the differences in the average values among populations in all measured characteristics are statistically significant ($p<0.05$). The LSD test did not show a clear separation of homogeneous groups, which was also demonstrated by the dendrogram cluster analysis. The results of this research can serve as an additional knowledge of the variability of beech in Serbia. If they are combined with other analyzes (morphological, anatomical, and molecular) they can contribute to solving specific problems in forestry related to this species.

***Keywords*:** common beech, morphological characteristics, cupules, natural populations, variability

INTRODUCTION

Beech (*Fagus sylvatica* L) is one of the most common and most important broadleaf species in Europe (VON VUEHLISCH, 2008; HOUSTON DURRANT *et al*., 2016). In Serbia, beech forests are characterized by a wide distribution area in terms of

elevation (they ranges from 40 to 2100 m.a.s.l.) (JOVANOVIĆ, CVJETIĆANIN, 2005) and account for 29.3% of forest cover and 40.5% of total tree volume (BANKOVIĆ *et al*., 2009). Given its importance, beech is a widely studied species from various aspects, both regionally (MIŠIĆ, 1957; IVETIĆ, 2009; STOJNIC *et al*., 2012; STOJNIC, 2013; SIJAČIĆ-NIKOLIC *et al*., 2013; BALIAN *et al*., 2015; IVETIĆ *et al*., 2018; KERKEZ JANKOVIĆ *et al*., 2019; NONIĆ *et al*., 2019) and globally (DENK, 1999; GÖMÖRY *et al*., 1999; DENK and MELLER, 2001; DENK *et al*., 2005; GÖMÖRY *et al*. 2007; CVRČKOVÁ *et al*., 2017; MÜLLER *et al*., 2019). The long-standing problem is the definition of the taxonomic status of beech in its southeastern part of the European range of distribution. To define taxonomic categories within the genus Fagus, among other things, morphological indicators have been used (CZECZOTT, 1933; MIŠIĆ, 1957; JANKOVIĆ, 1970). The morphological characteristics of beech cupules are very systematically significant (DENK and MELLER, 2001), and characterized by great polymorphism, especially in shape, length and width of the valve, petiole length and base shape (MIŠIĆ, 1955, 1957; IVETIĆ *et al*., 2018). However, there are only a few studies engage in variability of beech cupules shapes and dimensions (MISIC 1955, 1957; DENK 1999; DENK 2003; DENK *et al*. 2005; IVETIĆ *et al*. 2018).

This paper aims to determine the inter-population variability of beech at the level of 12 natural populations from Serbia based on the morphometric characteristics of beech cupules. Given the geographical location of the populations, one of the goals is to determine is there an unique pattern in the spatial distribution of beech in this area.

MATERIALS AND METHODS

The beech cupules were collected in early October 2018 from 12 natural populations of different elevations (300 to 1350 m.a.s.l.): Fruska gora (FG), Južni Kučaj (JK), Boranja (BR), Stara planina (SP), Rudnik (RU). Goč (GC), Severni Kučaj (SK), Jelova gora (JG), Kukavica (KU), Jastrebac (JB), Golija (GL), Javor (JV) (Figure 1). The site selection was made based on current knowledge about the beech provenance regions in Serbia (IVETIĆ, 2009).

From each population, 120 normally developed cupules were randomly selected for the analyses. Five morphological characteristics were analyzed, of which four were measured: length of longest valve (LV), width of longest valve at the widest part (WV), length of cupule without petiole (LC), length of petiole (LP) and one derived: „connecting piece" (CP = LC-LV) (Figure 2).

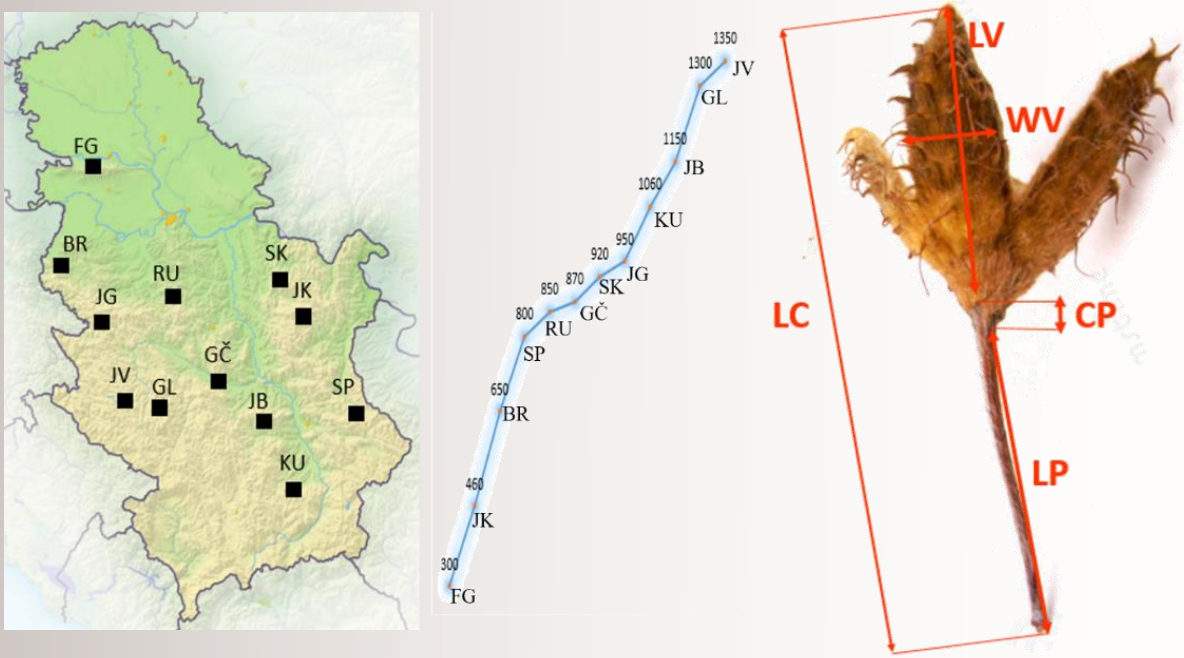

Figure 1. Spatial distribution (left) and elevation (m) (right) of the studied populations: Fruška gora (FG), Južni Kučaj (JK), Boranja (BR), Stara planina (SP), Rudnik (RU), Goč (GČ), Severni Kučaj (SK), Jelova gora (JG), Kukavica (KU), Jastrebac (JB), Golija (GL), Javor (JV).

Figure 2. Cupules measured properties: length of the longest valve (LV), width of the longest valve at the widest part (WV), length of cupule without the petiole (LC), length of the petiole (LP) and one derived: connecting part (CP).

The morphological characteristics of the cupules are defined by descriptive statistical indicators: arithmetic means (X), standard deviation (SD), coefficient of variation (CV%). One-factor analysis of variance (ANOVA) was used to determine variability between populations. If statistically significant differences were found between populations, additional testing was done by the Fisher Test (LSD). Cluster analysis was used to determine distances between populations. The dendrogram of the cluster analysis was based on Euclidean distances using the UPGMA method (Unweighted Pair Group Average Method). All the above statistical analyses were performed using the statistical program STATISTICA 7.0 (StatSoft Inc. 2004).

RESULTS AND DISCUSSION

The results of the descriptive statistical analysis (X, SD, CV%) and the results of the LSD test (groups of a-g) are shown in Table 1.

Table 1. Descriptive statistics for the measured morphological characteristics of the cupules

Population	Descriptive indicator	Morphological characteristics (mm)				
		LV	WV	LC	LP	CP
BR	$X^{LSD}\pm$ SD	20.29 $^{e}\pm$2.06	10.29 $^{bc}\pm$1.38	25.23 $^{de}\pm$2.36	9.39 $^{d}\pm$2.83	4.94 $^{b}\pm$1.51
FG		19.37 $^{bc}\pm$2.44	10.57 $^{cde}\pm$1.53	24.41 $^{bc}\pm$2.37	7.18 $^{a}\pm$1.96	5.04 $^{b}\pm$1.68
GČ		19.91 $^{cde}\pm$2.16	11.27 $^{g}\pm$2.07	24.97 $^{cd}\pm$2.58	8.13 $^{bc}\pm$3.61	5.06 $^{b}\pm$1.78
GL		19.50 $^{bcd}\pm$2.60	10.27 $^{bc}\pm$1.80	25.35 $^{de}\pm$3.18	10.36 $^{ef}\pm$2.89	5.86 $^{c}\pm$1.53
JB		21.76 $^{f}\pm$2.35	11.15 $^{fg}\pm$2.18	26.86 $^{f}\pm$2.55	13.15 $^{g}\pm$5.23	5.10 $^{b}\pm$1.81
JV		20.00 $^{de}\pm$1.99	10.84 $^{def}\pm$1.50	25.10 $^{cd}\pm$2.19	7.93 $^{ab}\pm$3.89	5.10 $^{b}\pm$1.87
JG		19.24 $^{b}\pm$3.95	9.83 $^{a}\pm$1.78	25.89 $^{e}\pm$5.08	8.05 $^{abc}\pm$2.45	6.65 $^{b}\pm$1.91
JK		21.45 $^{f}\pm$2.95	10.45 $^{cd}\pm$1.33	27.24 $^{f}\pm$3.50	11.15 $^{f}\pm$5.09	5.79 $^{c}\pm$1.99
KU		19.31 $^{b}\pm$1.66	11.14 $^{fg}\pm$2.01	24.19 $^{b}\pm$2.13	10.90 $^{f}\pm$4.50	4.88 $^{b}\pm$1.53
RU		20.43 $^{e}\pm$2.06	10.76 $^{def}\pm$1.28	25.32 $^{de}\pm$2.53	8.40 $^{bc}\pm$2.86	4.89 $^{b}\pm$1.73
SK		21.18 $^{f}\pm$1.42	10.90 $^{efg}\pm$1.70	25.87 $^{e}\pm$1.90	9.47 $^{de}\pm$3.16	4.69 $^{ab}\pm$1.67
SP		17.95 $^{a}\pm$1.36	9.88 $^{ab}\pm$1.26	22.36 $^{a}\pm$1.78	8.85 $^{cd}\pm$2.35	4.42 $^{a}\pm$1.63
Total		20.03±2.56	10.61±1.73	25.23±3.05	9.41±3.90	5.20±1.82
BR	CV %	10.17	13.39	9.36	30.15	30.56
FG		12.65	14.42	9.71	27.28	33.27
GČ		10.86	18.33	10.32	44.44	35.09
GL		13.32	17.49	12.56	27.86	26.10
JB		10.78	19.53	9.48	39.78	35.39
JV		9.96	13.80	8.74	49.10	36.71
JG		20.51	18.05	19.62	30.50	28.69
JK		13.77	12.73	12.86	45.65	34.53
KU		8.62	18.03	8.82	41.25	31.41
RU		10.08	11.94	9.99	34.07	35.35
SK		6.73	15.57	7.33	33.34	35.58
SP		7.55	12.77	7.96	26.57	36.90
Total		12.77	16.34	12.11	41.45	34.92

Legend: X - Mean; CV - coefficient of variation; SD - standard deviation; LSD - homogeneous groups designated from a to g; LV - valve length; WV - valve width; LC - length of cupule without petiole; LP - petiole length; CP - „connecting piece" ; Fruska gora (FG), Južni Kučaj (JK), Boranja (BR), Stara planina (SP), Rudnik (RU). Goč (GC), Severni Kučaj (SK), Jelova gora (JG), Kukavica (KU), Jastrebac (JB), Golija (GL), Javor (JV)

The mean values of all measured parameters are approximate to those already reported in the literature (MIŠIĆ, 1957; DENK, 1999; DENK, 2003; DENK *et al*., 2005; IVETIĆ *et al*., 2018). The low values of the coefficient of variability (CV<10%) are characterized by the properties of the length (LV) and the width (WV) of the valve and the length of the cupule (LC), while higher values are characterized by the length of the petiole (LP) and the length of the "connecting piece" (CP). The coefficient of variability (CV) indicates that the most variably measured characteristic is the length of the petiole (LP = 41.45%), which also has been confirmed in other studies (MIŠIĆ, 1957; IVETIĆ *et al*., 2018), while the least variable is the length of the cupule (LC = 12.11%).

In the biological sciences, the optimal values of the coefficient of variation are considered to be in the interval: 5% ≤ CV≤ 30%, and that the properties of the CV values over 30% do not show statistically significant differences (MIĆIĆ, BOSANČIĆ, 2012). However, according to the one-factor analysis of variance (Table 2), for all observed characteristics between populations, there are significant statistical differences at the significance level of p <0.05. The length of the „connecting piece" (CP) has the value of CV = 34.92% and is characterized by a clear grouping into three groups according to the LSD test (Table 1), while the other characteristics did not show a clear separation of homogeneous groups. According to the values of the „connecting piece", the first "group" (a) consists of the population of Stara planina, which also stands out in a separate group according to the characteristics of the length of the cupule, width, and length of the valve. At the transition between "a" and "b" group is the population of Severni Kučaj. Group "b" consists of the populations Boranja, Fruška gora, Goč, Jastrebac, Javor, Jelova gora, Kukavica, and Rudnik. While group "c" consists of the populations Južni Kučaj and Golija.

Table 2. Results of one-factor analysis of variance (ANOVA)

Source	**Between populations**			**F**	**p**
Trait	**SS**	**df**	**MS**		
LV	1532.47	11	139.315	25.21	**0.00**
WV	308.388	11	28.0353	9.96	**0.00**
LC	2113.70	11	192.155	24.26	**0.00**
LP	3855.17	11	350.47	27.74	**0.00**
CP	490.43	11	44.5845	14.96	**0.00**

The results of the cluster analysis show that the populations of Javor and Goč are grouped at the smallest genetic distance, making one homogeneous group with the populations of Rudnik, Boranja, Severni Kučaj, Fruška gora, as well as the Golija population. The second homogeneous group consists of the populations of Jastebac and Južni Kučaj and the third is Jelova gora and Stara planina populations (Figure 3). Based on the obtained results, it can be concluded that grouping of populations based on the morphological characteristics of the cupules, is not geographically conditioned.

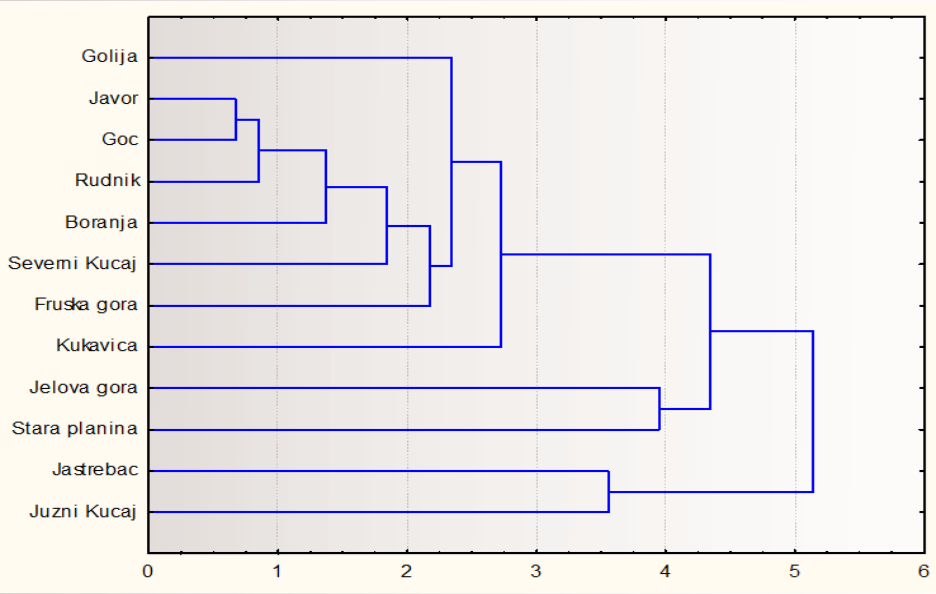

Figure 3. Dendrogram of cluster analysis based on Euclidean distances

CONCLUSION

Based on all the facts mentioned above, it can be concluded that there are statistically significant differences between the studied populations for the all studied morphological characteristics and that the variability is large. However, the results of analysis of variance, must be taken with caution, since the measured differences are not necessarily conditioned only by the gene pool of the population, but they are most often the result of the interaction of the gene pool and environmental conditions.

The mean values of the all measured characteristics are approximate to those already available in the literature. Therefore, it would be necessary for future studies to examine variability based on the descriptive characteristics of the cupules (base shape, stipule appearance, number and shape of valves).

Based on the obtained results of the morphometric analysis of the cupules it is not possible to determine existence of unique patterns in the spatial distribution of beech in this area.

This study can serve as an addition to current knowledge about the variability of beech populations in Serbia and knowledge about the variability of cupules in general. If combined with the results of other analyzes (morphological, molecular, anatomical, phenological) they can contribute to solving specific problems related to this species in taxonomy and forestry.

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REFERENCES

Ballian, D, Jukić, B, Balić, B, Kajba, D, von Wuehlisch, G (2015). Phenological variability of European beech (*Fagus sylvatica* L.) in the international provenance trial, Journal of the Forestry Society of Croatia, 11-12, (521-533).

Banković, S, Medarević, M, Pantić, D, Petrović, N (2009). National forest inventory of the republic of Serbia, Ministry of Agriculture, Forestry and Water Management of Serbia, Forest Directorate, Belgrade, (81-84).

Cvrčková, H, Máchova, P, Poláková, L, Trčková, O (2017). Evaluation of the genetic diversity of selected *Fagus sylvatica* L. populations in the Czech Republic using nuclear microsatellites, Journal of Forest Science, 63, (53–61).

Czeczott, H (1933). Studium nad zmiennoscia lisci bukow: *Fagus orienralis* Lipsky, *Fagus sylvatica* L. i form przejsciowych. Part 1, Roczn Polsk Tow Dendrol 5 (44-121).

Denk, T (1999). The taxonomy of *Fagus* in western Eurasia. 2: *Fagus sylvatica* subsp. *sylvatica*, Feddes Repert, 110, (381-412). doi:10.1002/fedr.19991100510

Denk, T (2003). Phylogeny of Fagus L. (*Fagaceae*) based on morphological data, Plant Systematics and Evolution, 240(1-4), (55-81).

Denk, T, Grimm, G, Hemleben, V (2005). Patterns of molecular and morphological differentiation in *Fagus*: Phylogenetic implications, American Journal of Botany, 92, (1006-1016).

Denk, T, Meller, B (2001). Systematic significance of the cupule/nut complex in living and fossil *Fagus*, International Journal of Plant Sciences, 162(4), (869-897).

Gömöry, D, Paule, L, Brus, R, Zhelev, P, Tomović, Z, Gračan, J (1999). Genetic differentiation and phylogeny of beech on the Balkan Peninsula. Journal of Evolutionary Biology 12, (746–754).

Gömöry, D, Paule, L, Vyšný, J (2007). Patterns of allozyme variation in Western Eurasian *Fagus*, Botanical Journal of the Linnean Society, 154, (165-174).

Houston Durrant, T, de Rigo, D, Caudullo, G (2016). *Fagus sylvatica* and other beeches in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz, J, de Rigo, D, Caudullo, G, Houston Durrant, T, Mauri, A (Eds.), European Atlas of Forest Tree Species. Publ. Off. EU, Luxembourg, (94-95).

Ivetić, V (2009). Delineation regions of provenances of beech in Serbia by the spatial analysis of genetic diversity. Ph.D. Thesis. University of Belgrade – Faculty of Forestry, Belgrade, Serbia.

Ivetić, V, Kerkez, I, Denić, I, Devetaković, J (2018). Variability of beech cupules in Serbia, Reforesta 5, (27-32). doi: https://dx.doi.org/10.21750/R EFOR.5.05.51

Janković, M (1970). Ред *Fagales* Фам. *Fagaceae* Род *Fagus* [Ord. *Fagales*, Fam. *Fagaceae* Gen. *Fagus*] In: Josifović, M. (ed.): Флора Србије 2 [The Flora of Serbia, Volume 2]. Serbian Academy of Sciences and Arts, Department of Natural and Mathematical Sciences. Belgrade, Serbia (69-74).

Jovanović, B, Cvjetićanin, R (2005). Taxonomy, morphology and distribution of the Moesian beech (*Fagus moesiaca* /DM/Cz) in Serbia. Beech (*Fagus moesiaca* /DM/Cz) in Serbia, Publishers: Association of Forestry Engineers and Technicians of Serbia, and Faculty of Forestry, University of Belgrade, Serbia, (75-82).

Kerkez Janković, I, Nonić, M, Devetaković, J, Ivetić, V, Šijačić-Nikolić, M, Aleksić, J. M (2019). Technical overview of nuclear microsatellites for *Fagus* sp., and their utility in *F. sylvatica* from the central Balkans (Serbia), Scandinavian Journal of Forest Research, 1-12. https://doi.org/10.1080/02827581.2019.1623305

Mićić, M, Bosančić, B (2012). Variability and Variation Coefficients in Biological and Agricultural Experimental Research, Agroknowledge Journal, Vol. 13(3), (331-342). doi: 10.7251/AGRSR1203331M

Mišić, V (1955). Principi i metode u istraživanju sistematike bukve u Jugoslaviji [Principles and methods in the research work of systematics of beech in Yugoslavia]. – Zbornik radova Instituta za ekologiju i biogeografiju, SANU, 6(1), (3-14).

Mišić, V (1957). Varijabilitet i ekologija bukve u Jugoslaviji [The variability and ecology of the beech in Yugoslavia]. – Posebno izdanje, Biološki institut NR Srbije, Beograd.

Müller, M, Lopez, P, A, Papageorgiou, A, C, Tsiripidis, I, Gailing, O (2019). Indications of Genetic Admixture in the Transition Zone between *Fagus sylvatica* L. and *Fagus sylvatica* ssp. *orientalis* Greut. & Burd., Diversity, 11(6), (1-11). https://doi.org/10.3390/d11060090

Nonić, M, Čortan, D, Batalo, T, Šijačić-Nikolić, M (2019). Comparative analysis of morphological characteristics of beech leaves from the European provenance trial, Bulletin of the Faculty of Forestry, 119, (145-174). https://doi.org/10.2298/GSF1919145N

Šijačić-Nikolić, M, Milovanović, J, Nonić, M, Knežević, R., Stanković D (2013). Leaf morphometric characteristics variability of different beech provenances in juvenile development stage, Genetika, Vol. 45(2), (369-380).

StatSoft Inc. (2004) "Statistica" Data Analysis Software System, version 7.

Stojnić, S (2013). Variability of anatomical, physiological and morphological traits of different European beech provenances in Serbia, Ph.D. Thesis. University of Belgrade – Faculty of Forestry, Belgrade, Serbia.

Stojnić, S, Orlović, S, Pilipović, A, Vilotić, D, Šijačić-Nikolić, M, Miljković, D (2012). Variation in leaf physiology among three provenances of European beech (*Fagus sylvatica* L.) in provenance trial in Serbia, Genetika, Vol. 44 (2), (341-353).

von Wuehlisch, G (2008). EUFORGEN Technical Guidelines for genetic conservation and use for European beech (*Fagus sylvatica*), Bioversity International, Rome, Italy.

THE TWO-LEVEL MARKER ASSISTED SELECTION IN BC_2 GENERATION OF THE CONVERSION OF STANDARD MAIZE LINES TO THEIR QPM VERSION

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ABSTRACT

Quality Protein Maize (QPM) is nutritionally enhanced maize. To shorten the period required for development of QPM hybrids through the conventional method of backcrossing, marker assisted selection (MAS) is being used. After a successful conversion of one commercial maize inbred line to its QPM counterpart for growing in temperate climate, four commercial Maize Research Institute (MRI) inbreds, chosen for marker assisted introgression of the quality protein trait, and their BC_2 progenies were subjected to two-level selection procedure. First, BC_2 plants were analyzed with *opaque2* (*o2)* specific molecular markers to identify heterozygotes. Second, the selected heterozygotes were screened with SSR markers to identify genotypes with the highest recovery of recurrent parent's genome (RPG). The specific markers identified 100 out of 192 plants (52%) as heterozygous. Genetic similarity values between parental lines and their BC_2 heterozygous progenies were in the range from 0.77 to 0.99 (77-99% RPG). The highest proportion of RPG was found in L_1 (93-99%) and the lowest in L_3 progenies (77-89%). Average values for the RPG content ranged from 83.9 to 95.8%. Progenies with RPG above 95% were selfed to produce BC_2F_2 plants which will be subjected to foreground selection. This time selection will be focused on homozygous recessive individuals, given that the presence of *opaque2* gene in the homozygous recessive state is the aim of the QPM selection. Finally, those *o2o2* genotypes will be screened for biochemical and phenotypic traits to confirm their nutritional and agronomical superiority.

Keywords: Maize, Marker assisted selection (MAS), *opaque2*, Quality Protein Maize (QPM)

INTRODUCTION

The discovery of *opaque2 (o2)* maize mutants (MERTZ *et al*., 1964) initiated the beginning of the breeding for improved protein quality in maize. This mutation has been

used as a source for genetic improvement of the nutritional value of maize proteins. Agronomically acceptable and nutritionally improved *opaque2* types (Quality Protein Maize -QPM) were created through conventional breeding programs by interdisciplinary research team in the International Maize and Wheat Improvement Center (CIMMYT), Mexico (VILLEGAS *et al*., 1992). In comparison to normal maize, QPM differs in protein quality because it contains double the amount of Lys and Try, the two amino acids deficient in maize proteins (PRASANNA *et al*., 2001; PANDA *et al*., 2010).

Marker assisted selection (MAS) has been increasingly used in maize protein quality improvement programs (BABU *et al*., 2005, DANSON *et al*., 2006; GUPTA *et al*., 2013). Foreground selection enables maintenance of recessive genes without the need for progeny testing in each generation of selection, as homozygous and heterozygous plants can be distinguished using gene-specific SSR markers. Also, DNA markers in background selection accelerate recurrent parent's genome (RPG) recovery.

Marker assisted selection contributes immensely to the conventional breeding. The tremendous benefits of the combined approach, as pointed out in MIAH *et al*., (2015), are: competent foreground selection for the locus of interest, effective background selection for the recovery of recurrent parent's genome, reduced linkage drag adjacent to the introgressed locus, prompt breeding for the development of new genotypes with favorable traits.

As a result of a breeding program at MRI, one commercial maize inbred line was converted to its QPM counterpart for growing in temperate climate (KOSTADINOVIC *et al*., 2014; KOSTADINOVIC *et al*., 2016). The aim of this research was to examine the efficiency of molecular markers for direct selection of the target gene (foreground selection), as well as for fast recovery of recurrent parent's genome (background selection) in BC_2 generation of this conversion process.

MATERIAL AND METHODS

Plant material

The QPM version of one commercial MRI inbred line was used as the donor parent (DP) of the favourable allele of the *opaque2* gene. Four MRI commercial inbred lines, components of the leading MRI hybrids, were used as the recurrent parents (RP_1, RP_2, RP_3 and RP_4). The conversion process is given in Figure 1. F_1 plants were pollinated with reccurent parent line to generate BC_1 progeny. The BC_1 plants heterozygous for the gene specific phi057 and umc1066 locus were selected for backcrossing. A two-level selection procedure was carried out in BC_2 generation. First, the individuals were screened with *opaque2* specific molecular markers to identify the heterozygotes. Second, these selected heterozygotes were screened with SSR markers distributed throughout the maize genome. Genotypes with the highest recovery of recurrent parent’s genome were selfed to produce BC_2F_2 seeds.

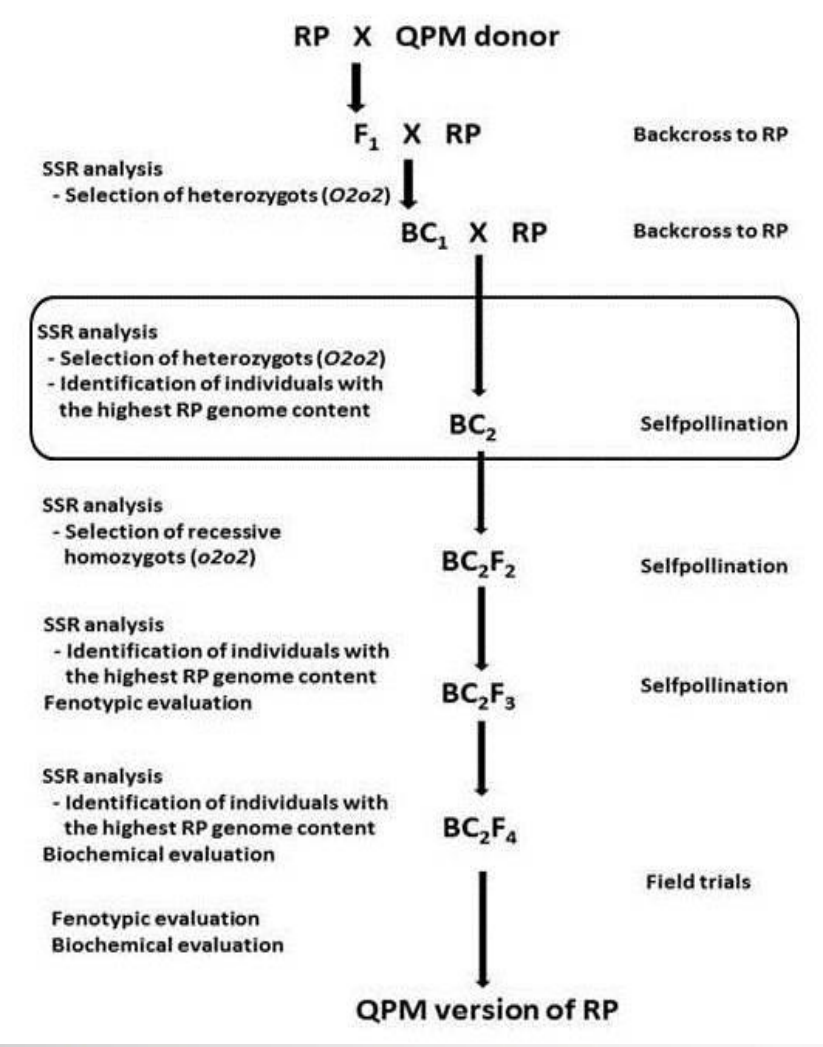

Figure 1. Scheme of MAS for conversion of standard maize inbred line to its QPM version.

DNA extraction

Genomic DNA was isolated from the kernel bulk according to DOYLE and DOYLE (1987) from the four-weeks-old plants. Bulks were prepared by pooling an equal amount of leaf tissue taken from 20 leaves per line. The concentration and the quality of the DNA was determined using biospectrometer BioSpectrometer kinetic (Eppendorf, Germany).

Foreground selection

Two gene specific SSR markers (phi057 and umc1066) were employed in foreground selection for the incorporation of *opaque2* into recepient maize lines (Table 1). Polymerase chain reaction was carried out in 20 μL reaction volume containing: 1×DreamTaq Green Buffer (Thermo Scientific, USA), 200 μM dNTP (Thermo Scientific, USA), 0.25 μM primers, 1U DreamTaq DNA Polymerase (Thermo Scientific, USA) and 20 ng DNA template. Amplifications were performed in thermocycler

Biometra TProfessional Standard 96 (Biometra, Germany) with the following program: an initial denaturation at 94ºC/2min, followed by 40 cycles each of denaturation at 94ºC/1min, annealing at 60ºC/2min and extension at 72ºC/2min with final elongation at 72° C for 10 min. The amplified fragments were resolved by 8% polyacrylamide gel electrophoresis on small format vertical gel system (Mini Protean Tetra-Cell, BioRad, USA). After staining with ethidium bromide, they were visualized under UV transilluminator and documented in gel documentation system BioDocAnalyze (Biometra, Germany). The size of the amplification products was determined comparing to the 50 bp molecular weight ladder (Thermo Scientific, USA).

Table 1. The set of SSR markers used in foreground selection for the opaque2 gene

Primer	Sequence
phi057 F	5'-CTCATCAGTGCCGTCGTCCAT-3'
phi057 R	5'-CAGTCGCAAGAAACCGTTGCC-3'
umc1066 F	5'-ATGGAGCACGTCATCTCAATGG-3'
umc1066 R	5'-AGCAGCAGCAACGTCTATGACACT-3'

Background selection

For the background selection, SSR analysis was done with 30 polymorphic markers spanning over the whole genome, selected from the maize database (www.maizegdb.org). Polymerase chain reaction (PCR) was carried out in 25 µL reaction volume containing: 1×DreamTaq Green Buffer (Thermo Scientific, USA), 200 µM dNTP (Thermo Scientific, USA), 0.5 µM of each primer, 1U DreamTaq DNA Polymerase (Thermo Scientific, USA) and 20 ng DNA template. The following touch-down program in the thermocycler Biometra TProfessional Standard 96 (Biometra, Germany) was performed: an initial denaturation at 95ºC/5min, followed by 15 cycles each of denaturation at 95ºC/30 s, annealing at 63.5ºC/1min (-0.5ºC/cycle) and extension at 72ºC/1min; another 22 cycles of 95ºC/30 s, 56ºC/1min and 72ºC/1min with final elongation at 72° C for four min. The PCR products were separated by electrophoresis on 8% polyacrylamide gel, with 20 bp molecular weight ladder (Thermo Scientific, USA) as a marker. After staining with ethidium bromide, they were photographed under UV light using Biometra BioDocAnalyze gel documentation system (Biometra, Germany). SSR profiles were converted into a binary matrix based on the presence (1) or the absence (0) of a specific allele. Genetic similarity (GS) was calculated in accordance with DICE (1945): GSij = 2a/2a+b+c; where: a is the number of fragments present in both variety i and j (1,1), b is the number of fragments present in i and absent in j (1,0), c is the number of fragments absent in i and present in j (0,1). Marker data analyses were performed using statistical NTSYSpc2 program package (ROHLF, 2000).

RESULTS AND DISCUSSION

The first level of selection procedure in BC_2 generation was identification of heterozygous plants with *opaque2* specific molecular markers phi057 and umc1066. Out of 192 plants, 100 (52%) were identified as heterozygous, which is in accordance with the expected Mendelian ratio of 1 *O2O2*: 1 *O2o2* in backcross generations. Out of these 100 heterozygous individuals, 30 originated from RP_1, 24 from RP_2, 20 from RP_3 and 26 from RP_4. Figure 2 shows the co-dominant nature of marker umc1066 that can distinguish homozygous and heterozygous genotypes. Lanes 3, 5, 8, 10, 11, 12 and 13 were heterozygous (*O2o2*) and lanes 4, 6, 7 and 9 were dominant homozygous individuals (*O2O2*).

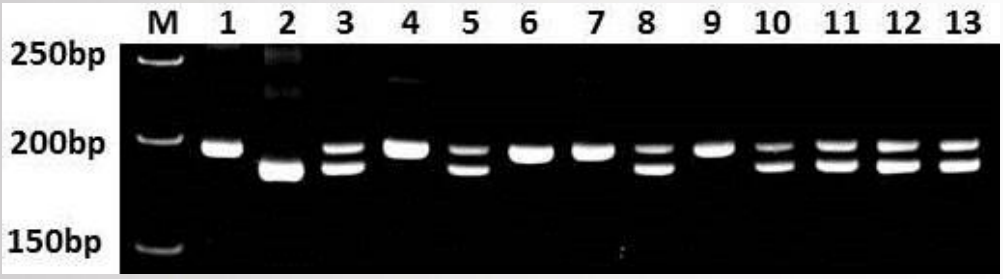

Figure 2. SSR profile of BC_2 individual plants detected with opaque2 specific marker umc1066. M: 50 bp DNA ladder, 1: standard line (recurrent parent), 2: QPM line (donor parent), 3-13: BC_2 individuals.

The second level of selection procedure in BC_2 generation was background selection performed on previously identified heterozygous plants (*O2o2*). Genetic variability between these individuals and their recurrent parents was analyzed with SSR markers distributed over the maize genom. As stated in MIAH *et al*., (2015), monomorphic markers bear no value in selection work since this type of marker is not able to distinguish the two parental genotypes. Markers that showed polymorphism were used in backcross generation. Total number of alleles detected with 30 informative markers was 39 for RP_1, 48 for RP_2, 68 for RP_3 and 61 for RP_4, average being 1.3, 1.6, 2.27 and 2.03, respectively. These values are somewhat lower than those previously reported in maize inbreds (BANTE and PRASANNA, 2003; LEGESSE *et al*., 2007; KOSTADINOVIC *et al*., 2018). The higher number of alleles per locus in other studies can be explained by the fact that analyses were performed on a larger number of different maize genotypes or by the use of a larger number of SSR markers in the analysis (MEHTA *et al*., 2017).

Genetic similarity values between the recurrent parents and their corresponding BC_2 progenies, calculated using Dice coefficient, ranged from 0.77 to 0.99 (77-99% RPG). The highest proportion of RPG was found in RP_1 (93-99%) and the lowest in RP_3 progenies (77-89%). Average values for the RPG content ranged from 83.9 to 95.8%. Similar percentages of parental genome renewal in the BC_2 generation have been reported in other studies (BABU *et al*., 2005; GUPTA *et al*., 2013; SINGH and RAM, 2014; THAKUR *et al*., 2014).

There was a great acceleration of recipient genome recovery in the present study. Theoretically, the proportion of the RPG after *n* generations of backcrossing is given by $(2^{n+1} - 1)/2^{n+1}$ (COLLARD *et al*., 2005). In our case, 66% of progenies had RPG above this theoretical value. Also, the RPG value of 99% was achieved in a few individuals, what is the value theoretically achieved in BC_6 generation. Fast recovery of RPG was attained probably due to the genetic similarity between donor and recipient lines, as well as the absence of linkage between the target gene and nearby genes from the donor parent and/or random genetic recombination.

Our study showed that molecular markers are efficient in reducing the time and resources involved in selection process. Selected heterozygous individuals with the highest RPG values were self-pollinated to produce BC_2F_2 plants. DNA samples from BC_2F_2 progenies will be collected and subjected to the foreground selection before flowering to identify the progenies that attained homozygosity at *o2* locus. Finally, those *o2o2* genotypes will be screened for biochemical and phenotypic traits to verify their nutritional and agronomical superiority.

CONCLUSIONS

Co-dominant nature of the polymorphism exibited by the phi057 and umc1066 primers enables their utility in MAS program to successfully discriminate between homozygotes and heterozygotes. Using polymorphic SSR markers in background selection, heterozygotes with the highest percentage of recurrent parent's genome were successfully identified. Our study confirmed the efficiency of molecular markers in determination of the success rate of genomic regions transfer (foreground selection), as well as the recovery rate of the recurrent parent’s genome (background selection).

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REFERENCES

Babu R., Nair S.K., Kumar A., Venkatesh S., Sekhar J.C., Singh N.N., et al. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor Appl Genet. 111:888-897.

Bantte, K., Prasanna B.M. (2003). Simple sequence repeat polymorphism in quality protein maize (QPM) lines. Euphytica, 129: 337-344.

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005): An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica, 142: 169-196.

Danson, J., Mbogori M., Kimani M., Lagat M., Kuria A., Diallo A. (2006). Marker assisted introgression of *opaque2* gene into herbicide resistant elite maize inbred lines. Afr J Biotechnol. 5(24):2417-2422.

Dice L.R. (1945): Measures of the amount of ecologic association between species. Ecology, 26: 297-302.

Doyle J.J., Doyle J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull, 19: 11-15.

Gupta H.S., Babu R., Agrawal P.K., Mahajan V., Hossain F., Thirunavukkarasu N. (2013). Accelerated development of quality protein maize hybrid through marker-assisted introgression of *opaque-2* allele. Plant Breed. 118:77-82.

Kostadinovic M., Ignjatović-Micić D., Stanković G., Vancetović J., Ristić D., Mladenović Drinić S. (2014). Genetic and biochemical characterisation of parental inbred lines in marker assisted selection for quality protein maize. Genetika, 46(2): 579-590.

Kostadinovic M., Ignjatović-Micić D., Vancetović J., Ristić D., Bozinovic S., Stanković G., Mladenović Drinić S. (2016). Development of High Tryptophan Maize Near Isogenic Lines Adapted to Temperate Regions through Marker Assisted Selection - Impediments and Benefits PLoS ONE, 11(12): e0167635. doi:10.1371/journal.pone.0167635.

Kostadinovic M., Ignjatovic-Micic D., Vancetovic J., Ristic D., Obradovic A., Stevanovic M., Mladenovic drinic S. (2018). Parental polymorphism analysis in marker assisted selection for β-carotene rich maize. Proceedings of the IX International Agricultural Symposium "Agrosym 2018", Jahorina, Bosnia and Herzegovina, October 4-7, 333-338.

Legesse, B.W., Myburg A.A., Pixley K.V., Botha A.M. (2007). Genetic diversity of African inbred lines revealed by SSR markers. Hereditas, 144: 10-17.

Mehta B., Hossain F., Muthusamy V., Baveja A., Zunjare R., Jha S. K., Gupta H. S. (2017). Microsatellite-based genetic diversity analyses of *sugary1*-, *shrunken2*- and double mutant- sweet corn inbreds for their utilization in breeding programme. Physiology and Molecular Biology of Plants, 23(2): 411-420.

Mertz E.T., Bates L.S., Nelson O.E. (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm, Science, 145: 1470-1473.

Miah G., Rafii M.Y., Ismail M.R., Puteh A.B., Rahim H.A., Latif M.A. (2015). Recurrent parent genome recovery analysis in a marker-assisted backcrossing program of rice (*Oryza sativa* L.) Comptes Rendus Biologies, 338: 83-94.

Panda A.K., Raju M.V.L.N., Rama Rao S.V., Lavanya G., Pradeep Kumar Reddy E., Shyam Sunder G. (2010). Replacement of normal maize with quality protein maize on performance, immune response and carcass characteristics of broiler chickens. Asian Australian Journal of Animal Sciences, 23: 1626-1631.

Prasanna B.M., Vasal S.K., Kassahun B., Singh KN. (2001). Quality protein maize. Current Science, 10: 1308-1319.

Rohlf F.J. (2000). NTSYS-pc Numerical Taxonomy and Multivariate Analysis System version 2.1. Owner's manual.

Singh R., Ram L. (2014). DNA Aided Introgression of *Opaque-2* Allele for Development of Quality Protein Maize. International Journal of Science and Research, 3(11): 3006-3011.

Thakur V.V., Tiwari S., Tripathi N. (2014). Studies on Genome Recovery Percentage for *Opaque2* Introgressed in Backcross Population for Marker Assisted Selection of Quality Protein Maize. Trends in Biosciences, 7(15): 1897-1901.

Villegas E., Vasal S.K., Bjarnason M. (1992). Quality protein maize - What is it and how was it developed. In: Mertz ET, editor. Quality Protein Maize. St. Paul, MN, USA: Am Assoc Cereal Chem; 27-48.

PHENOTYPIC AND MOLECULAR CHARACTERIZATION TOWARDS GENETIC PURITY ASSESSMENT OF MAIZE INBREDS

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ABSTRACT

Maintenance of genetic purity and uniformity of maize inbred lines and hybrids is a prerequisite for successful production and placement of commercial hybrid seed on the market. The aim of this study was to compare different marker types' efficiency regarding genetic purity assessment. Morphological characterization of three maize inbred lines was performed according to the UPOV (*Union Internationale pour la Protection des Obtentions Végétales*) markers in three-year field experiment. For uniformity and stability testing, 16 measured (MS) agro-morphological traits were evaluated. According to observed MS traits based upon three-way Analyses of Variance (ANOVA), all three inbred lines exhibited an acceptable degree of uniformity, while stability was more pronounced in L2 and L3 than in L1 inbred line. In parallel, 12 informative SSR markers were used for genetic purity analysis. Initial molecular marker analysis of the genotypes was done on bulked seed samples. Two out of 12 SSR markers revealed possible problems with genetic purity of L1 and L2 genotypes. Since for these genotypes two bands were scored upon visualization of PAGE electrophoresis, analyses were repeated with the two mentioned SSR markers on individual seeds of each genotype. The electrophoretic pattern of L1 individual seeds revealed its' impurity, with upper or lower band present. Electrophoregram of L2 individuals showed the presence of two bands in each seed, the same as in the bulk, which confirms its genetic homozigosity. Based on the results obtained, certain discrepances in genetic purity assessment were revealed, suggesting higher independence of molecular markers regarding environmental conditions.

***Keywords*:** stability, uniformity, morphological markers, SSRs, *Zea mays* L.

INTRODUCTION

One of the most important quality criteria required for successful hybrid seed production is genetic purity. To ensure maximum kernel set and high levels of genetic purity during hybrid seed production, much effort has always been directed towards

managing the process (BHAT *et al.*, 2017). Continued usage of morphological data to describe important genotypes indicates that those data retain popularity as descriptors of plant species (GALOVIĆ *et al*., 2006). Morphological markers provide an expensive, time consuming but reliable method for routine screening of a large number of accessions (CAROVIĆ *et al.*, 2011). Traditionally, morphological comparisons have formed the basis for genetic purity evaluations. The descriptor for maize published by UPOV (*Union Internationale pour la Protection des Obtentions Végétales*), is one of several guidelines for morphological descriptors in order to standardize the morphological description for maize (LAW *et al*., 2011). Distinctness, uniformity and stability (DUS) tests are generally conducted according to the UPOV guidelines for individual species. The DUS testing of varieties is one of the requirements for their entry into the National List and/or for granting of Plant Breeders' Rights (YADAV and SINGH, 2010). While phenotypic observations depend on genetic sensitivities to environmental conditions, molecular data can give a direct access to most part of the genome, and, in the same time, overcome most of disadvantages of morphological and biochemical markers that can be useful to distinguish varieties and off types (CHAUDHARY *et al*. 2018). Molecular markers, as high precision, time and resources saving technology, not stage or tissue specific and not affected by the environment, has been used in crop breeding and genetic purity testing (DANIEL *et al*., 2012). Simple Sequence Repeats (SSR) are PCR-based markers used for genetic analysis. These microsatellites are co-dominant, able to detect a high number of alleles per locus, abundant and uniformly dispersed throughout the genome. The SSR markers are highly polymorphic and very informative molecular markers, of great importance for rapid assessment of hybrid and parental line seed purity (REDDY *et al*., 2015).

For this reason, the aim of this work was to compare the efficiency of morphological and molecular markers in determination of genetic purity for three maize inbred lines evaluated.

MATERIALS AND METHODS

Three maize inbred lines (L1, L2 and L3) obtained from the Basic Seed Department of Maize Research Institute Zemun Polje, Serbia (44°52´N, 20°19´E, 81 m asl) were evaluated for distinctness, uniformity and stability according to the UPOV markers in three-year field trial (from 2016 to 2018).

Each maize inbred line was sown in four rows, spaced 0.75 m apart, with 20 plants per row, in two densities (D-30cm and D-40cm) and two sowing dates (ten-day interval between each sowing), in two replications, according to Complete Randomized Block Design.

Phenotypic variations regarding 16 measured (MS) agro-morphological traits

included – plant height (PH), plant height to upper most *node (PHN)*, number of leaves above upper most ear (NLAE), leaf length (LL), leaf width (LW), ear height (EH), length of main axis above the lowest lateral (LALB) and the highest lateral (LAHB) branch, are recorded in a particular developmental stage from two central rows on ten representative plants. Yield components – ear length (EL), ear diameter (ED), cob diameter (CD), kernel length (KL), kernel width (KW) and kernel thickness (KT), number of kernels per row (NKR) and number of rows per ear (NRE), were recorded on ten randomly chosen ears per replication. For each inbred, after manual harvesting and drying to 14% of water content, grain yield (GY) was calculated per plant basis. Three-way analyses of variance (ANOVA) were used for agro-morphological data analyzing and coefficient of variation (CV), as a measure of uniformity, was calculated for each trait and expressed in percentage.

DNA-pooled sampling strategy (bulk and single seed analyses) was used for SSR analysis. Each inbred was presented with ten plants. Genomic DNA was isolated from seed, using a modified CTAB procedure (SAGHAI-MAROOF *et al*., 1984). A total of 12 informative SSR markers (phi 109275, umc 1448, phi 083, phi 102228, umc 1117, umv 1478, umc 1133, umc 1545, phi 233376, phi 015, bnlg1129, umc 1061) were chosen for the analysis (ISTA, 2018). PCR amplification reaction was carried out in 25 µl reaction volume containing DreamTaq™ Green PCR Master Mix (2X) (Thermo Scientific), 0.5µM primers and 50 ng of DNA. Amplification was conducted using the following touchdown cycling profile: an initial denaturation at 95°C/5 min, followed by 15 cycles each of denaturation at 95°C/30 s, annealing at 63.5°C/1 min (- 0.5°C/cycle) and extension at 72° C/1 min; another 22 cycles of 95°C/30 s, 56°C/1 min and 72°C /1 min were performed. Final elongation was at 72°C for 4 min. Amplified fragments were separated on 8% polyacrylamide gels with 20bp DNA marker ladder (Thermo Scientific) on a vertical gel system (Mini Protean Tetra-Cell BioRad) and stained with ethidium bromide. The gels were photographed using BioDocAnalyze (BDA) gel documentation system (Biometra, Germany) and SSR profiles for each primer were determined.

RESULTS AND DISCUSSION

The phenotype uniformity is a genotype ability to specify a phenotype consistency (FASOULA and TOLLENAAR, 2005). Uniformity is genetically controlled and is a key target for selective breeding in crops (TOKATLIDIS *et al*., 2010). By UPOV, when a variety has been shown to be uniform, it can also be considered as stable. However, the environment can induce changes in the individual's behavior at a morphological and/or physiological level (GRATANI, 2014). Generally, coefficient of variation (CV) is a measure applied to present variation in agro-morphological traits evaluated. In our study, the highest CV was observed for ear height (10.89%) in L1, and

the number of kernels per row (9.12) in L2 and (10.96%) in L3 inbred, respectively (Table 1). The lowest CV was recorded for cob diameter (1.12%) in case of L1, for kernel width (2.8%) in L2, i.e. ear diameter (2.26) in L3 inbred line. Since CV below 15% for any agro-morphological trait measured in field trial is considered as highly acceptable (KOZAK *et al*., 2013), inbred lines evaluated in our experiment exhibited high level of uniformity.

In addition, the results of ANOVA showed that the year (Factor A) had highly significant effect ($p \leq 0.001$) on observed genotypes for almost all agro-morphological traits (Table 1). Such a strong impact explains the fact that experiment was conducted during three years, highly varying in environmental conditions. Also, in interactions with other two factors, factor A had high influence on observed traits. In our study, 2017 was extremely dry, which was reflected through high reduction of PH in all three inbreds, and which is in line with reported findings of ÇAKIR (2004).

Plant density and planting pattern affected yield and morphological traits in a different manner (FARNIA and MANSOURI, 2015). In agronomic practices, plant density exerts a strong influence on maize growth, because of its competitive effect on both the vegetative and reproductive development (SINGH and CHOUDHARY, 2008). In our case, plant density (Factor B) had the biggest impact on the inbred L1, especially concerning the yield components, while L3 was affected the least. More pronounced effect of planting densities on yield components compared to morphological traits in all three inbreds is in line with YILMAZ *et al*. (2008), who reported that maize yield and yield components were significantly affected by planting patterns and plant densities. The values obtained for the majority of the observed traits were higher under lower plant density, which is in agreement with reports of MOOSAVI *et al*. (2012) and SHAFI *et al*. (2012).

In order to minimize negative effect of some abiotic and biotic stress on plant, sowing date can play a major role in determining seed germination, yield and its quality, and understanding the whole plant phenology in different environments (CHHETRI *et al*., 2018). Compared to other two factors, sowing date (Factor C) had a significant influence ($p \leq 0.01$) only on LALB and LAHB in L1 line, and low influence ($p \leq 0.05$) on KL in L2 and LALB in L3 inbred. Interaction AxB had the highest influence on L1 both for morphological traits and yield components, while L2 and L3 inbreds were affected only in 4 i.e. 2 yield components, respectively. Interaction AxC influenced mostly L3 inbred, and, to a lesser extent, L2 and L1. For all the inbreds, interaction BxC has shown little effect on all traits observed. The highest effect of AxBxC interaction, was noticed in inbred L1, while L2 and L3 varied only in one trait each.

Table 1. Significance for observed agro-morphological traits according to ANOVA for inbred lines evaluated

		Morphological traits									Yield components						
Inbred line	SV	PH	PHN	NLAE	LL	LW	EH	LALB	LAHB	EL	ED	CD	KL	KW	KT	NKR	NRE
L1	Factor A	***	***	ns	***	***	***	**	**	**	***	***	***	***	**	***	**
	Factor B	ns	ns	ns	*	ns	ns	*	*	**	***	***	*	ns	ns	**	*
	Factor C	ns	ns	ns	ns	ns	ns	**	**	ns	ns	ns	ns	ns	ns	ns	ns
	AxB	**	*	ns	*	ns	ns	**	*	*	**	***	ns	ns	ns	ns	ns
	AxC	ns	ns	ns	ns	ns	ns	*	*	*	ns	***	ns	ns	ns	ns	**
	BxC	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns
	AxBxC	ns	ns	ns	ns	ns	ns	ns	ns	*	**	*	***	ns	ns	ns	ns
	CV%	3.22	5.01	6.22	3.08	5.70	10.89	2.81	4.11	2.59	1.40	1.12	2.58	4.35	9.50	4.26	3.68
L2	Factor A	***	***	**	***	***	***	ns	ns	**	**	***	***	***	*	***	**
	Factor B	ns	ns	ns	ns	ns	ns	*	ns	*	ns	*	ns	*	ns	*	ns
	Factor C	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
	AxB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	***	***	*	ns
	AxC	ns	ns	ns	ns	ns	ns	***	ns	ns	ns	*	ns	*	ns	ns	*
	BxC	*	*	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	AxBxC	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	**	ns	ns	ns
	CV%	4.14	6.55	5.07	2.87	3.84	5.51	3.88	6.97	5.53	6.11	3.90	5.33	2.80	7.63	9.12	5.04
L3	Factor A	***	***	**	***	***	***	***	ns	**	***	***	***	***	**	*	ns
	Factor B	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns
	Factor C	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
	AxB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	*	ns	ns
	AxC	*	ns	ns	ns	**	ns	**	***	ns	ns	ns	ns	ns	**	ns	ns
	BxC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
	AxBxC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
	CV%	4.65	5.96	5.77	4.04	3.64	8.70	3.53	4.31	5.02	2.26	3.69	4.03	6.86	10.32	10.96	3.55

SV – source of variation; Factor A – year; Factor B – plant density; Factor C – sowing date; CV – coefficient of variation; P – plant height; PHN – plant height to upper most node; NLAE – number of leaves above upper most ear; LL – leaf length; LW – leaf width; EH – ear height; LALB – lowest lateral; LAHB – length of main axis above the highest lateral branch; EL– ear length; ED – ear diameter; CD–cob diameter; KL–kernel length; KW–kernel width; KT– kernel thickness; NKR– number of kernels per row; NRE– number of rows per ear,**,*** – significant at $p \leq 0.05$, 0.01 and 0.001 probability level, respectively; ns – non-significant.*

According to the results presented in Table 1, it could be concluded that all three inbred lines were quite uniform, while stability was more pronounced in L2 and L3 than in L1 inbred line the most uniform and stabile was L3 inbred, followed by L2 inbred, while L1 inbred varied the most, thus failing to meet, to some extent, the required level of uniformity and stability.

Since the morphological markers are labor intensive, time-consuming and highly influenced by the environmental conditions, nowadays, molecular markers are considered as the most reliable tool for genotypes' fingerprinting, assessing variation within parental lines and genetic purity testing (TAMILKUMAR *et al.*, 2009). Number of markers should be balanced with the accuracy of the genotype required for the objective. The number of markers to reach the necessary discriminative power depends on marker-type (dominant/co-dominant; bi-/multi-allelic), species and the quality of the marker performance (UPOV TG/2/7, 2009). For this reason, twelve informative SSR markers were used for the genetic purity analysis of three morphologically estimated inbred lines in order to compare the efficiency of morphological and molecular markers in determination of genetic purity.

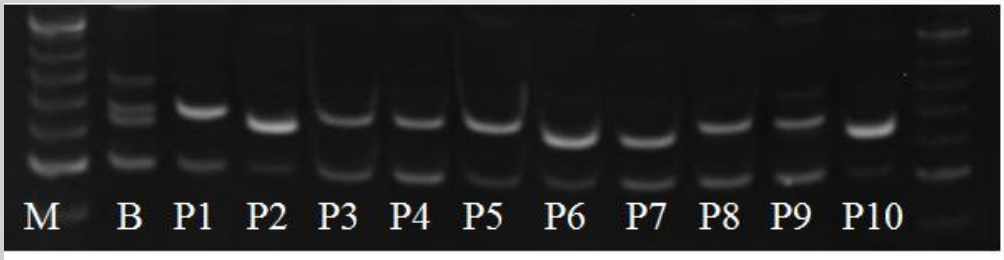

Figure 1. Electrophoregram of SSR analysis for L1 with phi102228.
M - 20 bp DNA ladder; B - seed bulk sample; P1-P10 - single seed samples.

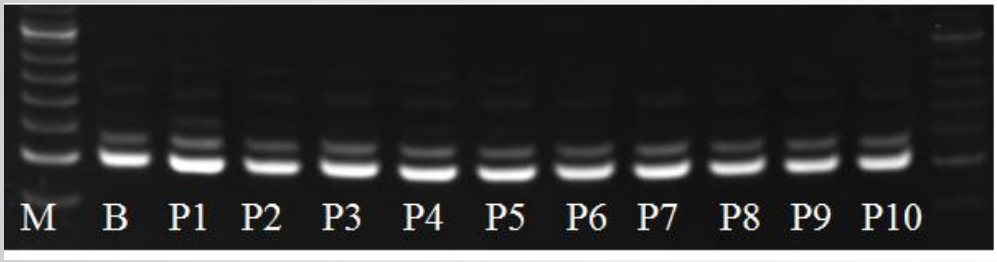

Figure 2. Electrophoregram of SSR analysis for L2 with umc1133.
M - 20 bp DNA ladder; B - seed bulk sample; P1-P10 - single seed samples.

The results of initial molecular marker analyses from bulk seed samples showed that two (phi102228 and umc1133) out of 12 SSR markers used, revealed possible heterozygosity (impurity) of L1 and L2 maize genotypes. Upon visualization of PAGE electrophoresis for these two inbreds, two alleles were scored. Analyses were repeated with the two mentioned SSR markers on ten individual seed samples of both genotypes.

Electrophoregram of L1 individual seeds (Figure 1) with upper or lower alleles present, revealed that this inbred line is not uniform. The electrophoretic pattern of L2 individuals (Figure 2) showed the presence of two alleles in each seed sample, the same as in the bulk samples, which confirms its genetic uniformity. Analyses of both types of seed samples, individual and bulk, showed the same results, which is in line with REIF *et al*. (2005). Genetic purity tested by SSR markers showed that L3 and L2 inbreds were genetically homozygous (pure), while L1 was heterozygous (impure).

CONCLUSIONS

Ascertaining genetic purity of inbred lines as parental components of maize hybrid is one of the most important quality control aspects in hybrid seed production. By estimation of variations in measured agro-morphological traits, all three inbred lines exhibited an acceptable degree of uniformity, while stability was more pronounced in L2 and L3 than in L1 inbred line. However, results obtained by SSR markers confirmed genetic purity only for L2 and L3 inbreds. Certain discrepances in estimation of genetic purity obtained by the two markers types could be the result of neutral nature of SSR markers, as markers uninfluenced by environmental or growth conditions.

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REFERENCES

Bhat, M.I., Gulzaffar, Dar Z.A., Lone, A.A., Shikari, A.B., Ali, G., Wani, M.A., Khan, G.H., Gazal, A., Lone, R.A. (2017). Genetic Purity Analysis in Maize under Temperate Conditions. International Journal of Current Microbiology and Applied Sciences, 6 (9), 2710 - 2722.

Çakir, R. (2004). Effect of water stress at different development stages on vegetative and reproductive growth of corn. Field Crops Research, 89 (1), 1 - 16.

Carović, K.S., Šalinović, A., Grdiša, M., Liber, Z., Kolak, I., Satovic, Z. (2011). Efficiency of morphological trait descriptors in discrimination of *Ocimum basilicum* L. accessions. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 145 (2), 298 - 305.

Chaudhary, S., Yashveer, S., Dinesh, Prajapati, D.R., Kharb, P., Kamboj, M.C. (2018). Utilization of SSR Markers for Seed Purity Testing in Popular Maize Hybrids. International Journal of Current. Microbiology and Applied Sciences, 7 (4), 1117 - 1126.

Chhetri S., Poudel A., Kandel B.Pd. (2018). Effect of sowing dates on grain yield and other agronomic traits of different maize inbred lines. International Journal of Scientific and Engineering Research, 9 (5), 428 - 434.

Daniel, I.O., Adetumbi, J.A., Oyelakin, O.O., Olakoj, S.A., Ajala, M.O., Onagbesan, S.O. (2012). Application of SSR Markers for Genetic Purity Analysis of Parental Inbred Lines and Some Commercial Hybrid Maize (*Zea mays* L.) American Journal of Experimental Agriculture, 2 (4), 597 - 606.

Fasoula, V.A., Tollenaar, M. (2005). The impact of plant population density on crop yield and response to selection in maize. Maydica, 50, 39 - 48.

Farnia, A., Mansouri, M. (2015). Study on Morphological Characteristics of Maize (*Zea mays* L.) Cultivars under Different Plant Densities. Indian Journal of Natural Sciences, 5 (30), 8391 - 8553.

Gilovich, V., Mladenović Drinić, S., Navalušić, J., Zlokolica, M. (2006). Characterization methods und fingerprinting of agronomicaly important crop species. Genetika-Belgrade, 38 (2), 83 - 96.

Gratani, L. (2014). Plant Phenotypic Plasticity in Response to Environmental Factors. Advances in Botany, 2014, 17 pages.

ISTA (2018): International Rules for Seed Testing. Chapter 8, Variety Testing. International Seed Testing Association, Bassersdorf, Switzerland

Kozak, M., Bocianowski, J. Rybinski, W. (2013). Note on the use of coefficient of variation for data from agricultural factorial experiments. Bulgarian Journal of Agricultural Science, 19 (4), 644 - 646.

Law, J.R., Anderson, S.R., Jones, E.S., Nelson, B.K., Mulaosmanovic, E., Smith J.S. (2011). Characterization of maize germplasm: comparison of morphological dataset compiled using different approaches to data recording. Maydica, 56 (2), 1710 - 1722.

Moosavi, S.G., Seghatoleslami, M.J., Moazeni, A. (2012). Effect of planting date and plant density on morphological traits, LAI and forage corn (Sc. 370) yield in second cultivation. International Research Journal of Applied and Basic Sciences, 3 (1), 57 - 63.

Reddy, D.C., Khandagale, K., Victorathisayam, T., Aswath, C., Singh, T.H. (2015). Genetic purity of interspecific hybrids of Solanummelongena and S. macrocarpon assessed using SSR markers International Journal of Vegetable Science, 21 (2), 111 - 117.

Reif, J., Hamrit, S., Heckenberger, M., Schipprack, W., Maurer, P.H., Bohn, M., Melchinger, A. (2005). Genetic structure and diversity of European flint maize populations determined with SSR analyses of individuals and bulks. Theoretical and Applied Genetics, 111 (5), 906 - 913.

Shafi, M., Bakht, J., Ali, S., Khan, H., Khan, M.A., Sharif, M. (2012). Effect of planting density on phenology, growth and yield of maize (*Zea mays* L.). Pakistan Journal of Botany, 44 (2), 691 - 696.

Singh, D. Choudhary, J. (2008). Effect of plant population and fertilizer levels on yield and economics of pop corn. Indian Journal of Plant Science, 78 (4), 370 - 371.

Tamilkumar, P., Jerlin, R., Senthil, N., Ganesan, K.N., Jeevan, R.J. Raveendran, M. (2009). Fingerprinting of Rice Hybrids and their Parental Lines using Microsatellite Markers and their Utilization in Genetic Purity Assessment of Hybrid Rice. Research Journal of Seed Science, 2 (3), 40 - 47.

Tokatlidis, I., Haş, V., Mylonas, I., Haş I., Evgenidis, G., Melidis, V., Copandean, A., Ninou, E. (2010). Density effects on environmental variance and expected response to selection in maize (*Zea mays* L.). Euphytica, 174 (2), 283 - 291.

UPOV TG/2/7. (2009). Guidelines for the conduct of tests for distinctness, uniformity and stability. International Union for the Protection of New Varieties of Plants, Geneva.

Yadav, V.K., Singh, I.S. (2010). Comparative evaluation of maize inbred lines (*Zea mays* L.) according to DUS testing using morphological, physiological and molecular markers. Agricultural Sciences, 1 (3), 131 - 142.

Yilmaz, K.K., Hoshin, V.G., Thorsten, W. (2008). A process-based diagnostic approach to model evaluation: Application to the NWS distributed hydrologic model. Water Resources Research, 44 (9), 1 - 18.

CHLOROPHYLL AND FLAVONOIDS CONTENT IN MAIZE INBREDS UNDER CONTRASTING WATER SUPPLY CONDITIONS

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ABSTRACT

In breeding of superior genotypes that are able to endure frequent and severe summer droughts, evaluation of leaf chlorophyll content, as an indicator of photosynthetic capability of plant tissues, growth and productivity, could assist. Likewise, evaluation of flavonoids status (such as anthocyanins and flavonols), as a class of specialized secondary metabolites with strong radical scavenging activity, contributes to mitigation of drought stress. Accordingly, the aim of this study was to determine whether the tolerance/susceptibility to drought stress can be attributed to the level of flavonoids and chlorophyll in maize leaves. For this purpose, five maize inbreds differing in drought tolerance were evaluated in two sets of field experiment: under well-watered and rain-fed conditions. At flowering, chlorophyll, flavonol and anthocyanin indices, as well as the nitrogen balance index (NBI) that represents the ratio between the mesophyll chlorophyll and epidermal flavone leaf contents, were analyzed. Also, anthesis-silking interval (ASI) as secondary trait relevant for drought tolerance and grain yield were determined. In drought tolerant lines, effect of water deficit was reflected through NBI and chlorophyll increase, followed by flavonoid content decrease. However, the opposite trend was noticed in drought susceptible maize inbreds. Under rain-fed conditions, anthocyanins content decreased or remained unchanged, except for highly pronounced increase in drought susceptible A632 inbred. Compared to well-watered set of field experiment, quite opposite trend in correlations between grain yield and indices measured was found under water deficit conditions. The results obtained indicated the activation of different metabolic pathways in defence against existing water stress.

Keywords: ASI, phenolic compounds, grain yield, water stress, *Zea mays* L.

INTRODUCTION

In temperate regions worldwide, the ongoing climatic changes cause frequent and severe summer droughts which seriously impaire maize grain yield. Activation of specific physiological and molecular responses, as plant mechanisms for acclimation and adaptation to challenging environmental conditions, leads to changes in plant metabolism in order to minimize stress-induced damage. Plants specifically alter gene

expression in a very diferent way that changes occurring in plants grown under defined stress conditions (water deficit or heat stress applied individually) (MITTLER and BLUMWALD, 2010). These alterations in gene expression result in a specific regulation of the metabolome depending upon the particular stress and the plant species (SUN *et al.*, 2016).

Plant metabolome comprises a huge diversity of metabolites with many different biological functions that is usually divided into primary metabolism, including polar metabolites such as carbohydrates, as direct products of photosynthesis and substrates of the energy metabolism, tricarboxylic acid cycle intermediates, and amino acids involved in protein synthesis or other cell processes like osmotic readjustment (CALDANA *et al*., 2011). Secondary metabolites such as phenylpropanoids and their derivatives are an important group of compounds essential for plant acclimation and survival to varying environmental conditions, including coumarins, lignin building blocks, flavonoids, anthocyanins and tannins (DJOUKENG *et al*., 2008). These, usually semi-polar compounds, have a variety of physiological roles, including ROS scavenging, enzyme activation, photoprotection and signal regulation (ZHAO *et al*., 2005; ARBONA *et al*., 2013).

Water stress may not simply trigger acclimation mechanisms, but also result in various damages (URBAN *et al*., 2017). Most damage-related parameters that can be measured in the field are indicators of leaf chlorophyll content. Water deficit may cause damage to the oxygen-evolving center coupled with photosystem II (PSII), leading to inhibition of the photosystem functionality reflected through inactivation of the PSII reaction centers (ZLATEV, 2009), which may eventually lead to reactive oxygen species (ROS) generation, as well as photoinhibition and oxidative damage (GILL and TUTEJA, 2010). The analysis of chlorophyll content, as photosynthetic trait, is considered as an important approach for evaluating the integrity of the internal apparatus during photosynthetic process within a leaf and provides a rapid and accurate technique of detecting and quantifying plants tolerance to drought stress.

According to the findings mentioned above, the aim of this study was *i*) to determine whether the tolerance/susceptibility to water stress can be attributed to the status regarding chlorophyll, flavonols and anthocyanins in maize leaves, and *ii*) to evaluate the relationship between photosynthetic *i.e.* secondary metabolic indices and grain yield, as an indicator of plant productivity, achieved under well-watered and rain-fed conditions.

MATERIALS AND METHODS

Three inbred lines from Maize Research Institute „Zemun Polje“ gene bank mini-*core* set (L1–L3) and two public drought susceptible inbred lines (B73 and A632) were evaluated.

The experiment was carried out in 2017 in Zemun Polje, Serbia (44°52´N, 20°19´E, 81 m a.s.l.). Inbred lines were grown in two sets of field experiment: under well-watered set (considered as the optimal condition – OC) and under rain-fed experimental set (*i.e.* without irrigation, *e.g.* considered as water deficit condition). The plants of each maize inbred were sown in plot of two 4 m long rows, with an intra-row and inter-row separation of 0.40 m *i.e.* 0.75 m, respectively, with two plants per hill. A randomized complete block design with two replications was used in the experimental sets.

At flowering, chlorophyll index (Chl), flavonoid index (Flav), anthocyanin index (Anth) and the nitrogen balance index (NBI) as the ratio between the mesophyll chlorophyll and epidermal flavone leaf contents, were analysed. The measurements were conducted on the basal, middle and apical position of the uppermost ear leaf on twenty plants per genotype, using Dualex Scientific hand-held optical leaf clip sensor as non-destructive quantification of chlorophyll. In addition, the interval between anthesis and silking (ASI) as one of the most relevant secondary trait for drought tolerance was measured. The plants were harvested manually, and after drying to 14% of water content, the yield was determined and presented as the average grain yield per plant (g plant^{-1}).

Separately for both field experimental sets, Pearson's correlation coefficient was used for determination the relationship between indices measured and grain yield obtained.

RESULTS AND DISCUSSION

In the context of plant–water relations, the plant is a particularly complex system, due to its morphological plasticity and the physiological response in order to provide management strategies for optimum water supply and it's efficient use (BODNER *et al*., 2015). To achieve drought tolerance improvement of crop varieties through breeding, a set of reliable traits that can be rapidly and relatively inexpensively screened is needed (SILVA *et al*., 2007).

Water deficit has been reported to diminish root nutrient uptake and translocation to the leaves (FAROOQ *et al.*, 2009). In this context, non-destructively measured leaf Chl content was reported to be a surrogate for leaf nitrogen (N) in plant photosynthetic capacity and productivity research and is an indicator of N nutrition of crops (TREMBLAY *et al*., 2010), while NBI is more of an indicator of C/N allocation changes due to N-deficiency than a measure of leaf nitrogen content *per se* (CARTELAT *et al*., 2005).

The percentage of change in measured indices was displayed in Figure 1. Compared to well-watered field experimental set, water deficit caused the reduction of NBI for 8.8% and 33.8% in drought susceptible B73 and A632 inbred lines.

However, under water deficit stress, drought tolerant inbreds exhibited increase of NBI, ranging from 19.2% in L2 to 34.6% in L3, *i.e.* 38.0% in L1, respectively.

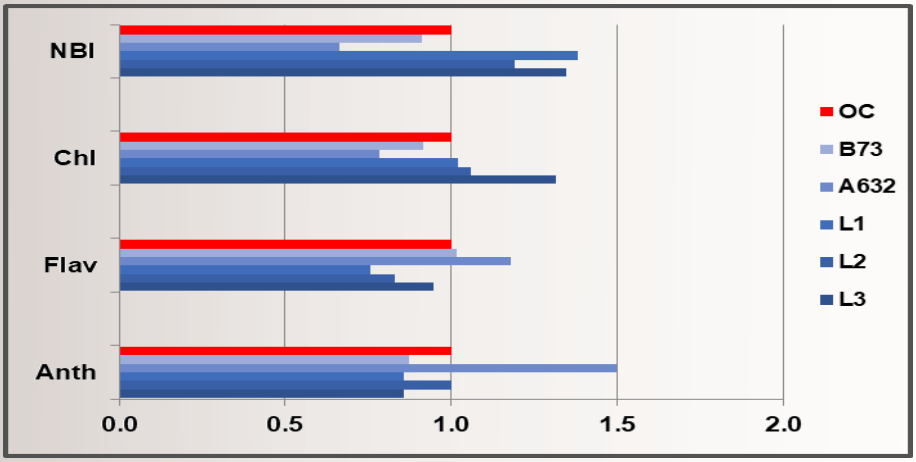

Figure 1. The percentage of changes for indices measured under water deficit stress compared to the optimal conditions evidenced in drought tolerant (L1, L2 and L3) and drought susceptible (B73 and A632) inbreds. OC – values measured under optimal conditions given as 1 (100%).

In response to water deficit, the same trend was observed for Chl index. Namely, drought susceptible inbreds exhibited decrease of Chl for 8.4% (inbred B73) and for 21.7% (line A632). Previously it was reported that the majority of Chl lost from maize leaves subjected to water stress is lost from the mesophyll cells being farther removed from the vascular supply of water than the bundle sheath cells, thus developing greater cellular water deficits which lead to a higher Chl loss. Likewise, the mesophyll chloroplasts may be subject to greater Chl losses because they contain more of the light-harvesting Chl–protein complexes of thylacoid membranes that are labile even under mild water deficit conditions (SHAO *et al.*, 2016). Because the light-harvesting Chl–protein complex is a major intrinsic membrane component, it is likely that losses in this component may lead to perturbation of the chloroplast membrane structural organization and to a reduction in the efficiency of the membrane dependent electron transport of photosynthesis. Opposite to drought susceptible genotypes evaluated in the experiment described herein, the highest increase of Chl for 31.7% was found in drought tolerant L3, followed by slight Chl index increase for 2.2% and 6.1% in L1 and L2 lines. Our results revealed that the components of the photosynthetic apparatus could be damaged in drought sensitive genotypes, and that the drought tolerant genotypes could possess the adaptability to decrease/evade impairment resulted from water stress. It may be suggested that genetic differences exist in the reaction of the photosynthetic apparatus to drought, and that in drought tolerant genotypes the photosynthetic process has a higher tolerance to water deficit stress, which is in line with the reports from study on barley (RONG-HUA *et al.*, 2006).

It was reported that flavonoids, a class of specialized secondary metabolites, including flavonols and anthocyanins with strong radical scavenging activity, contributed to the mitigation of oxidative and drought stress in plants (AGATI *et al*., 2012). In our experiment, opposite trend regarding relative flavonols content was observed between drought sensitive and drought tolerant lines (Figure 1). Namely, drough susceptible lines exhibited Flav index increase of 1.8% in B73 and 18.1% in A632 line. Among drought tolerant inbreds, L1 expressed the most pronounced decrease in Flav of 24.4%, followed by L2 and L3 (Flav reduction of 16.9% and 5.2%), respectively. Our results are in agreement with SELMAR (2008), who reported that plants under stress conditions often produce a higher degree of flavonoids compared to non-stressed plants. Also, data obtained from the study on a series of Arabidopsis lines overaccumulating and lacking flavonoids under MYB overexpression showed that the flavonoid-specific MYB-overexpressing plants did not show enhanced drought tolerance through ABA-induced abiotic stress response mechanisms under the unstressed condition (NAKABAYASHI *et al*., 2014). These findings might suggest more preserved mechanisms for preventing water loss from the plant tissue in drought tolerant lines evaluated in our study.

Under water stress, nitrogen deficit impairs photosynthetic function and efficiency and decreases the levels of Calvin Cycle enzymes, which resulted in induced or enhanced accumulation of foliar anthocyanin in leaves of many plant species (PENG *et al*., 2008). Compared to well-watered field experimental set, water deficit stress in our experiment resulted in decreased (for 12.6% in drought sensitive B73 and for 14.3% in L1 and L3 drought tolerant lines) or unchanged Anth index (in L2 line), respectively. However, highly pronounced increase in Anth index of 50.0% was observed in drought susceptible A632 inbred (Figure 1). Since A632 exhibited the highest reduction of both Chl index and NBI, possible explanation could be that N stress caused by water deficit triggered the expression of genes encoding enzymes associated with anthocyanin biosynthesis in this drought susceptible genotype (STEYN *et al*., 2002).

Impact of water deficit on maize grain yield depends on the synchrony between the stress and the developmental stage. If it occurs during flowering, as it was the case in our study, yield losses are the most pronounced. Anthesis-silking interval is an indicator of plant health and nutritional and water status of silks, ovules and kernels (OURY *et al*., 2016). The more pronounced variation in ASI (even up to four days) was observed in drought susceptible A632 line (Figure 2). The four-day interval difference could be considered as discontinuous, disturbed pollination, since it occured under severe water deficit that disturb physiological role of silk up to 40.0% (CEROVIĆ *et al*., 2014). Water deficit resulted in grain yield decrease in all the genotypes evaluated, ranging from,

26.0% in A632 to 46.6 in B73 drought susceptible lines. In drought tolerant L1, L2 and L3 inbreds, grain yield decline was as followed: 11.2%, 3.0% and 13.6% respectively(Figure 2). In addition, widely reported negative correlation between ASI and grain yield (COOPER *et al*., 2014) was in line with our findings, particurly regarding drought susceptible inbreds ($p \leq 0.001$).

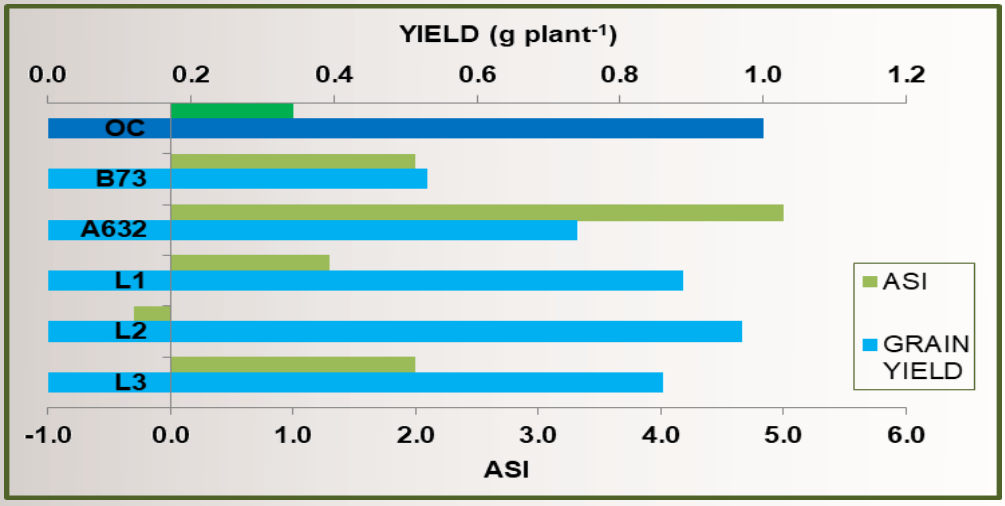

Figure 2. The percentage of changes for ASI and grain yield under water deficit stress compared to the optimal conditions evidenced in drought tolerant (L1, L2 and L3) and drought susceptible (B73 and A632) inbreds. OC – values measured under optimal conditions given as 1 (100%).

Flowering period in maize is the most vulnerable phenophase to water deficit. However, during the grain-filling period, water and nitrogen availability determine the extent to which sink and source contribute to yield formation, and limited resource availability will mainly result in source restrictions by reducing current photosynthesis and less by sink limitations (OVEYSI *et al*., 2010). Since chloroplasts comprise more than 70.0% of nitrogen (N) taken up from the environment, grain yield and its quality are dependent on chloroplast breakdown, as a consequence of water stress, and remobilization of nitrogen. Better preserved previously mentioned light-harvesting Chl-protein complex of thylacoid membranes in chloroplasts, which is rapidly catabolized under stress conditions, suggests strongly that it may be a readily mobilized source of amino nitrogen for maintenance protein synthesis, as well as a source of carbon skeletons for energy production during stress (SHAO *et al*., 2016) towards higher grain yield. These findings are in line with highly significant and positive correlations between grain yield and NBI, *i.e.* Chl index ($p \leq 0.01$ for NBI, *i.e.* $p \leq 0.001$ for Chl index, respectively) otained under severe water stress (Table 1).

Table1. Pearson's correlations between indices and grain yield obtained under different water supply conditions

	NBI	Chl	Flav	Anth
GY under OC	-0.610^{ns}	-0.615^{ns}	0.252^{ns}	0.379^{ns}
GY under WD	0.798**	0.880***	-0.652*	-0.252^{ns}

*Chl – Chlorophyll index; Flav – Flavonol index; Anth – Anthocyanin index; NBI – Nitrogen balance index; GY – grain yield; OC – optimal conditions; WD – water deficit; *,**,*** – significant at P≤0.05, 0.01 and 0.001 probability level, respectively; ns – non-significant*

CONCLUSION

The distribution of Dualex's measured values for Chl index and especially for NBI among the maize genotypes evaluated in this study is consistent with their drought tolerance/susceptibility classification. This is confirmed by their highly significant and positive correlation ($p \leq 0.001$ for Chl index, *i.e.* $p \leq 0.01$ for NBI, respectively) with obtained grain yield, as a measure of plant productivity under severe water deficit stress. In addition, observed quite opposite trend in correlations between grain yield and all the indices measured found under water deficit conditions compared to well-watered field experimental set, indicated the activation of different metabolic pathways in defence against existing severe water stress.

ACKNOWLEDGEMENT

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REFERENCES

Agati, G., Azzarello, E., Pollastri, S., Tattini, M. (2012). Flavonoids as antioxidants in plants: location and functional significance. Plant Science, 196, 67 - 76.

Arbona, V., Manzi, M., de Ollas, C., Gómez-Cadenas, A. (2013). Metabolomics as a tool to investigate abiotic stress tolerance in plants. International Journal of Molecular Sciences, 14 (3), 4885 - 4811.

Bodner, G., Nakhforoosh, A., Kaul, H-P. (2015). Management of crop water under drought: a review. Agronomy for Sustainable Development, 35 (2), 401 - 442.

Caldana, C., Degenkolbe, T., Cuadros-Inostroza, A., Klie, S., Sulpice, R. et al. (2011). High-density kinetic analysis of the metabolomic and transcriptomic response of Arabidopsis to eight environmental conditions. Plant Journal, 67 (5), 869 - 884.

Cartelat, A., Cerovic, Z.G., Goulas, Y., Meyer, S., Lelarge, C., Prioul, J.-L., Barbottin, A., Jeuffroy, M.-H., Gate, P., Agati, G., Moya, I. (2005). Optically assessed contents of leaf polyphenolics and chlorophyll as indicators of nitrogen deficiency in wheat (*Triticum aestivum* L.). Field Crops Research, 91 (1), 35 - 49.

Cerović, R., Pajić, Z., Filipović, M., Fotrić-Akšić, M., Radičević, S., Nikolić, D., Đorđević, M. (2014). Pollen germination and pollen tube growth in ZP maize lines. Genetika-Belgarde, 46 (3), 935 - 948.

Cooper, M., Messina, C.D., Podlich, D., Radu Totir, L., Baumgarten, A., Hausmann, N.J., Wright, D., Graham, G. (2014). Predicting the future of plant breeding: complementing empirical evaluation with genetic prediction. Crop and Pasture Science, 65 (4), 311 - 336.

Djoukeng, J.D., Arbona, V., Argamasilla, R., Gomez-cadenas, A. (2008). Flavonoid profiling in leaves of citrus genotypes under different environmental situations. Journal of Agricultural and Food Chemistry, 56 (23), 11087 - 11097.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., Basra, S.M.A. (2009.) Plant drought stress: Effects, mechanisms and management. Agronomy for Sustainable Development, 29 (1), 185 - 212.

Gill, S.S., Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry, 48 (12), 909 - 930.

Mittler, R., Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. Annual Review of Plant Biology, 61, 443 - 462.

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., Matsuda, F., Kojima, M., Sakakibara, H., Shinozaki, K., Michael, A.J., Tohge, T., Yamazaki, M., Saito, K. (2014). Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. Plant Journal, 77 (3), 367 - 379.

Oury, V., Tardieu, F., Turc, O. (2016). Ovary apical abortion under water deficit is caused by changes in sequential development of ovaries and in silk growth rate in maize. Plant Physiology, 171 (2), 986 - 996.

Oveysi, M., Mirhadi, M.J., Madani, H., Nourmohammadi, G., Zarghami, R., Madani, A. (2010). The impact of source restriction on yield formation of corn (*Zea mays* L.) due to water deficiency. Plant Soil and Environment, 56 (10), 476 - 481.

Peng, M., Hudson, D., Schofield, A., Tsao, R., Yang, R., Gu, H., Bi, Y.M., Rothstein, S.J. (2008). Adaptation of Arabidopsis to nitrogen limitation involves induction of anthocyanin synthesis which is controlled by the NLA gene. Journal of Experimental Botany, 59 (11), 2933 - 2944.

Rong-hua, L., Pei-pol, G., Baumz, M., Grand, S., Ceccarelli, S. (2006). Evaluation of Chlorophyll Content and Fluorescence Parameters as Indicators of Drought Tolerance in Barley. Agricultural Sciences in China, 5 (10), 751 - 757.

Shao, R., Xin, L., Zheng, H., Li L., Ran, W., Mao, J., et al. (2016). Changes in chloroplast ultrastructure in leaves of drought-stressed maize inbred lines. Photosynthetica, 54 (1), 74 - 80.

Selmar, D. (2008). Potential of salt and drought stress to increase pharmaceutical significant secondary compounds in plants. Landbauforschung Volkenrode, 58 (1/2), 139 - 144.

Silva, M.A., Jifon, J.L., Da Silva, J.A.G., Sharma, V. (2007). Use of physiological parameters as fast tools to screen for drought tolerance in sugarcane. Brazilian Journal of Plant Physiology, 19 (3), 193 - 201.

Steyn, W.J., Wand, S.J.E., D. Holcroft, D.M., Jacobs, G. (2002). Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist, 155 (3), 349 - 361.

Sun, C.X., Li, M.Q., Gao, X.X., Liu, L.N., Wu, X.F., Zhou, J.H. (2016). Metabolic response of maize plants to multi-factorial abiotic stresses. Plant Biology, 18 (S1), 120 - 129.

Tremblay, N, Wang, Z, Belec, C. (2010). Performance of Dualex in spring wheat for crop nitrogen status assessment, yield prediction and estimation of soil nitrate content. Journal of Plant Nutrition, 33 (1), 57 - 70.

Urban, L., Aarrouf, J., Bidel, L.P.R. (2017). Assessing the Effects of Water Deficit on Photosynthesis Using Parameters Derived from Measurements of Leaf Gas Exchange and of Chlorophyll *a* Fluorescence. Frontiers in Plant Science, 8, 2068.

Zhao, J., Davis, L.C., Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnology Advances, 23 (4), 283 - 333.

Zlatev, Z. (2009). Drought-induced changes in chlorophyll fluorescence of young wheat plants. Biotechnology and Biotechnological Equipment, 23 (Supp 1), 438 - 441.

VARIABILITY OF MAIN NUTRIENTS IN MAIZE INBRED LINES CAUSED BY APPLICATION OF ORGANIC PEROXIDES

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ABSTRACT

Exogenous application of chemical elicitors to plants can be successfully used to reduce damage caused by abiotic stresses and consequently to enhance productivity. Different organic peroxides, applied foliarly as DMSO solution, were used in this study with the aim to examine variations in grain yield and main nutrient status, i.e. protein, starch and oil contents, of four maize inbred lines. Results showed that two genotypes reacted positively on applied treatments, achieving higher grain yields than control group, with differences up to 2-3 t ha^{-1}. In total, one of the applied peroxides expressed the highest impact on yield enhance. In terms of nutritive quality, the same treatment mostly increased the starch content. With regard to protein content, higher value was achieved by the same genotype which had higher grain yield, and for oil content, variations in results among treatments were insignificant and irregular. This indicates that synthetic elicitors, such as organic peroxides, could be used not only to increase grain yield, but also to modify grain nutritional quality in regard to genotype variability.

***Keywords*:** foliar treatment, chemical elicitors, grain, yield, nutritional quality

INTRODUCTION

Maize belongs to the three most widely grown crops and it is ranked first among the cereal crops grown throughout the world (WU *et al.*, 2019; VANCETOVIC *et al.*, 2014). It is one of the most important crops due to its wide uses as food, feed and as industrial material. Consequently, the main objective of the breeding program is to create hybrids, i.e. lines with improved properties, both in terms of quality and yield potential. From the quality characteristics, main nutrients in maize grain are starch, oil and protein. Starch is energy source, while protein and oil content are especially important traits at the maize market (MLADENOVIĆ DRINIĆ *et al.*, 2013a; YANG *et al.*, 2013; MLADENOVIC-DRINIC *et al.*, 2013b; AL-NAGGAR *et al.*, 2016).

According to PUNJ and HARYANA (2012) abiotic stresses such as drought, temperature, salinity, etc. that induce oxidative stress affect plant grow, productivity and quality. Maize mitigates induced damage through various mechanisms: activating enzymatic and nonenzymatic antioxidants, growth regulators and osmoprotectants. Many studies (AHMAD *et al.*, 2014; TRIVEDI *et al.*, 2018; AHMAD *et al.*, 2013) involved foliar application of different elicitors with the aim to overcome negative environmental impacts by stimulating one of these mechanisms. Exogenous treatment enhances maize antioxidant defense system, positively affecting the productivity and nutrient quality of grain as a result of lower degree of stress on the plant (CHATTHA *et al.*, 2015). It is reported that plant growth regulators improve photosynthate partitioning and grain development, thereby increasing plant tolerance to stress (WAQAS *et al.*, 2017). This research was aimed to examine elicitor effect of organic peroxides, with structure similar to plant growth hormones (MESAROVIĆ *et al.*, 2015), on yield and main nutrient status of four different maize inbred lines.

MATERIALS AND METHODS

The experiment was carried out to investigate elicitor effect of synthetic organic peroxides (belonging to mixed tetraoxanes) on grain yield and status of protein, starch and total oil in different maize inbred lines. Seeds of four maize inbred lines: L 355/99 (G1), L 76 B004 (G2), L 76 B036 (G3) and L 73 B003 (G4) were sown in randomized block design at field of Maize Research Institute „Zemun Polje“, Serbia. Each line was sown in two rows of 5 m in length, with four replications. In 5-8 leaves stage lines were treated foliarly with four organic peroxides (T1-T4), dissolved in DMSO solution, to assure their absorption by foliage. Two control groups had also been set up, one without spraying (C) and one treated with DMSO only (D).

After harvesting, grain yield was measured and calculated to 14% of moisture. The content of protein, starch and oil was determined on infrared analyzer (Infraneo, Chopin Technologies, France) and expressed as percentage of grain dry matter.

Results were analyzed using analysis of variance (ANOVA) and the significance of the treatments effect were determined by the Fisher’s least significant difference (LSD) test at $p = 0.05$. Evaluation of interdependence between genotypes and treatments was analyzed using principal component analysis (PCA). Statistical data were processed by SPSS 15.0 (IBM Corporation, Armonk, New York, USA) for Windows Evaluation version.

RESULTS AND DISCUSSION

G1 line provided higher yield in comparison to the other three lines (Table 1). However, treatments haven’t expressed effectiveness because treatment with DMSO, as

control, gave the highest yield. Foliar application of peroxides expressed good results in lines G2 and G3, achieving yields of 2-3 tons higher than control. One of the applied peroxides (T3) showed the highest impact on yield enhance. Results obtained in this research are in accordance with results obtained by AHMAD *et al.* (2014) in which mixture of ascorbic acid, salicylic acid and hydrogen peroxide applied on grain increase yield under stressful conditions. They explained observed effect through increased antioxidant activity which helped the maize crop to maintain relatively optimal photosynthesis under abiotic stress.

Table 1. Effect of different genotypes (G1-G4) and exogenous treatments (T1-T4) on grain yield (t ha^{-1}) of maize inbred lines

	G1	**G2**	**G3**	**G4**	**Average**
C	10.41	4.58	6.51	6.56	7.02
D	11.31	6.25	6.10	6.24	7.47
T1	10.01	6.57	6.06	6.39	7.26
T2	9.08	8.10	6.99	6.28	7.61
T3	10.88	7.24	7.35	5.87	7.83
T4	9.00	7.11	7.18	6.54	7.46
Average	10.12	6.64	6.70	6.31	7.44
$LSD_{0.05}$	T 1.44,	G 0.75,	T x G 1.68		

In terms of nutritive value, significant variability in starch and protein content was observed. Taking into account, genotype by treatment interaction, results for protein content (Table 2) showed that elicitors had positive influence only in G3, while in G1, G2 and G4 lines maximum values were recorded in control groups (C or D).

Table 2. Effect of different genotypes (G1-G4) and exogenous treatments (T1-T4) on protein content (expressed as % of grain DM).

	G1	**G2**	**G3**	**G4**	**Average**
C	10.29	10.98	9.92	9.54	10.18
D	10.61	10.94	10.23	9.46	10.31
T1	10.55	10.95	10.07	9.31	10.22
T2	10.10	10.47	10.32	9.27	10.04
T3	10.17	10.19	10.00	9.19	9.89
T4	10.22	10.23	10.32	9.13	9.98
Average	10.32	10.63	10.14	9.32	10.10
$LSD_{0.05}$	T 0.47,	G 0.20,	T x G 0.38		

Concerning the starch content (Table 3), one of applied peroxides (T3) raised starch level in three genotypes (up to the 0.54, 0.07 and 0.53%, for G1, G3 and G4, respectively). The same treatment also expressed the greatest influence on yield enhance (Table 1). Similarly, CHATTHA *et al*. (2015) reported greater yield and starch content by applying some of growth promoting substances, as well as enhanced protein content, which is opposite to our results. Exogenous treatments affect the tested parameters in different ways, depending on the type of applied elicitor and environmental conditions (TRIVEDI *et al.*, 2018; WAQUAS *et al.*, 2017), which is also confirmed in this research.

Table 3. Effect of different genotypes (G1-G4) and exogenous treatments (T1-T4) on starch and oil content of maize grain.

	Starch (%)[a]					Oil (%)[a]				
	G1	**G2**	**G3**	**G4**	**Average**	**G1**	**G2**	**G3**	**G4**	**Average**
C	68.93	70.15	69.70	71.50	70.07	3.67	3.57	3.93	3.50	3.67
D	69.00	68.17	68.97	71.27	69.35	3.67	3.63	3.83	3.23	3.59
T1	69.03	67.70	69.23	71.47	69.36	3.47	3.63	4.00	3.37	3.62
T2	69.10	69.37	69.47	71.53	69.87	3.60	3.70	3.87	3.30	3.62
T3	69.47	69.47	69.77	72.03	70.18	3.50	3.63	3.73	3.43	3.58
T4	69.30	69.53	69.53	71.50	69.97	3.50	3.90	3.93	3.37	3.68
Average	69.14	69.06	69.44	71.55	69.80	3.57	3.68	3.88	3.37	3.62
$LSD_{0.05}$	T 0.98, G 0.43, T x G 0.83					T 0.19, G 0.09, T x G 0.20				

[a]*Values are expressed as a percentage of grain dry matter.*

In contrast, T1 peroxide adversely affected content of starch in almost all maize inbred lines (Table 3). Among tested genotypes, G4 is the line with the highest starch level in all treatments and control groups, too.

For total oil content, differences at a significant level were obtained only among genotypes, whereas variations in results due to foliar treatment were insignificant and irregular (Table 3). This indicates that applied peroxides were ineffective for modification of oil content.

PCA

In order to evaluate the interaction among maize inbred lines, applied treatments and nutrient content, the PCA was used and indicated that first two axes were explained by 59.9% and 25.6% of total variability for examined parameters, respectively. Projection of variables are shown in Figure 1. and pointed that protein and starch content contributed mainly to PC1 (90.6% positively and 96.4% negatively, respectively), while grain yield to PC2 (86.4%). These results are in line with previous studies, where a negative correlation between starch content on the one side and protein and oil content

on the other side are confirmed (MLADENOVIĆ DRINIĆ *et al.*, 2013a; YANG *et al.*, 2013). All treatments applied on G1, as well as T2 application on G2, affected the yield, while starch content was altered by all applied treatments on G4. Similarly, applied foliar treatments influenced the oil content in G3, while in G2 line, protein and oil content varied mainly, depending on the treatments.

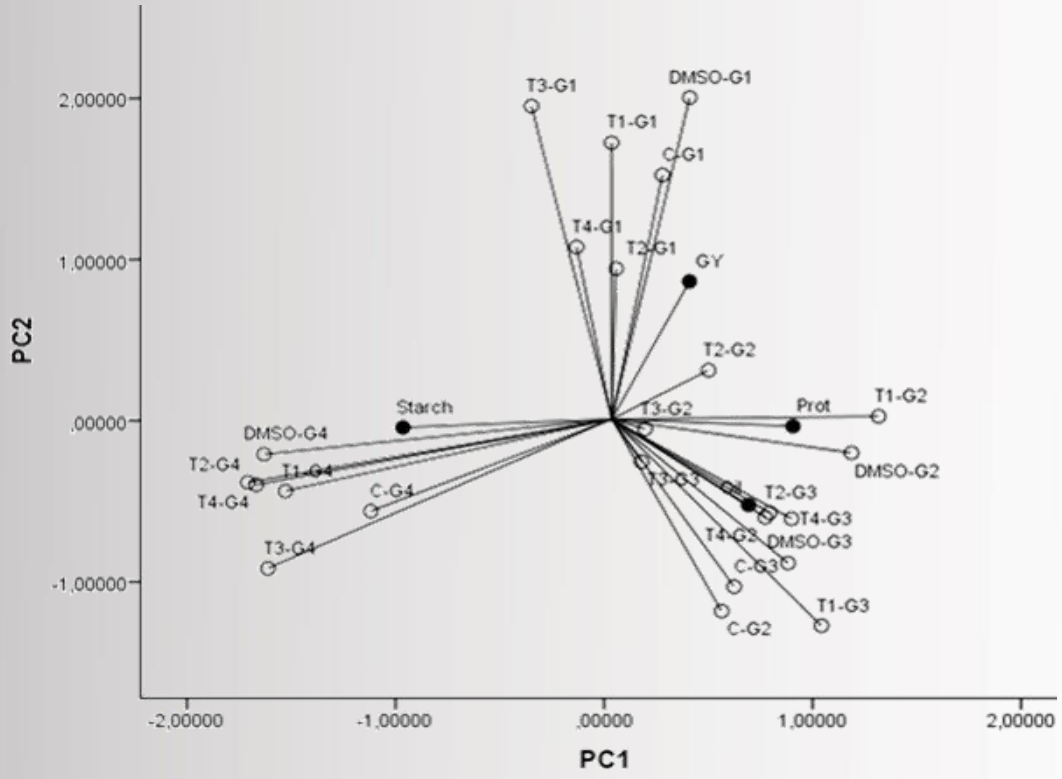

Figure 1. The obtained PCA plot for PC1 and PC2 components, showing the interaction among genotypes, elicitors and main nutrients.

CONCLUSION

Significant differences among genotypes were present for the yield, as well as content of main nutrients. G1 and G4 were genotypes with the highest average yield and starch content, respectively, whereas significant variations in protein and oil content were present among all genotypes.

According to the obtained results, exogenous treatment increased grain yield with differences up to 2-3 t ha^{-1} in G2 maize line. Foliar application of T3 peroxide provided the highest yield and starch content increase. Accordingly, T3 elicitor should be tested on a larger number of maize genotypes in order to confirm its benefits. In terms of protein content, elicitors have had positive influence only in G3 line, the same one with a higher grain yield. Applied peroxides didn't express significant impact on oil content.

This indicates that various elicitors, such as organic peroxides, could be used not only to increase grain yield, but also to modify grain nutritional quality, including variability among genotypes.

ACKNOWLEDGMENTS

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REFERENCES

Ahmad I., Basra S.M.A., Afzal I., Farooq M., Wahid A. (2013). Stand establishment improvement in spring maize through exogenous application of ascorbic acid, salicylic acid and hydrogen peroxide. International Journal of Agriculture and Biology, 15, 95–100.

Ahmad I., Basra S.M.A., Wahid A. (2014). Exogenous application of ascorbic acid, salicylic acid and hydrogen peroxide improves the productivity of hybrid maize under at low temperature stress. International Journal of Agriculture and Biology, 16, 825-830.

Al-Naggar A.M.M., Atta M.M.M., Ahmed M.A., Younis A.S.M. (2016). Heterosis and Combining Ability of Maize (*Zea mays* L.) Grain Protein, Oil and Starch Content and Yield as Affected by Water Stress. Archives of Current Research International, 4(4), 1-15.

Chattha M.U., Sana M.A., Munir H., Ashraf U., Ul-Haq, I., Zamir, S.I. (2015). Exogenous application of plant growth promoting substances enhances the growth, yield and quality of maize (*Zea may*s L.). Plant Knowledge Journal, 4(1), 1-6.

Mesarović J., Dragičević V., Mošić D., Opsenica D. (2015). Uticaj mešovitih 1,2,4,5-tetraoksana na rast klijanaca kukuruza. Kratki izvodi radova, Treća konferencija mladih hemičara Srbije, Beograd, 71.

Mladenović Drinić S., Dragičević V., Filipović M., Čamdžija Z., Stevanović M., Kovačević D. (2013a). Variranje sadržaja proteina, ulja i skroba u ZP inbred linijama kukuruza. Selekcija i semenarstvo, 19(2), 61-69.

Mladenovic-Drinic S., Dragicevic V., Filipovic M., Stojiljkovic M., Camdzija Z., Simic D., Kovacevic D. (2013b). Genetic variation of macro and microelements in grain of maize inbred lines. Book of Proceedings, Agrosym 2013, Jahorina, 173-177.

Punj S., Haryana N. (2012). Abiotic Stress: New Research. Nova Science Publishers, Inc, Hauppauge, N.Y.

Trivedi K., Anand K.G.V., Vaghela P., Ghosh A. (2018). Differential growth, yield and biochemical responses of maize to the exogenous application of *Kappaphycus alvarezii* seaweed extract, at grain-filling stage under normal and drought conditions. Algal Research, 35, 236-244.

Vancetovic J., Ignjatovic-Micic D., Bozinovic S., Babic M., Filipovic M., Grcic N., Andjelkovic V. (2014). Grain quality of drought tolerant accessions within the MRI Zemun Polje maize germplasm collection. Spanish Journal of Agricultural Research, 12(1), 186-194.

Waqas M.A., Khan I., Akhter M.J., Noor M.A., Ashraf U. (2017). Exogenous application of plant growth regulators (PGRs) induces chilling tolerance in short-duration hybrid maize. Environmental Science and Pollution Research International, 24(12), 11459-11471.

Wu Y., Wang L., Bian S., Liu Z., Wang Y., Lv Y., Cao Y., Yao F., Li C., Wei W. (2019). Evolution of roots to improve water and nitrogen use efficiency in maize elite inbred lines released during different decades in China. Agricultural Water Management, 216, 44–59.

Yang G., Dong Y., Li Y., Wang Q., Shi Q., Zhou Q. (2013). Verification of QTL for Grain Starch Content and Its Genetic Correlation with Oil Content Using Two Connected RIL Populations in High-Oil Maize. PLoS ONE, 8(1), e53770.

SURVIVING OXIDATIVE STRESS - NEW CELLULAR FUNCTIONS INVOLVED IN RECYCLING AND GENOME PROTECTION IN *USTILAGO MAYDIS*

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ABSTRACT

Ustilago maydis is a unicellular phytopathogenic fungus known for its extreme resistance towards UV and ionizing radiation. This feature selected *U. maydis* as a powerful model system for studying DNA repair and homologous recombination.

As a free-living and biotrophic microorganism infecting maize, *U. maydis* is exposed to various harsh environmental stresses throughout both of its life styles. *U. maydis* cell population subjected to severe stress could be preserved by efficient mechanism of DNA repairation. But powerful DNA repair system may not be the only cellular facility underlying genome protection and overall ecological success of *U. maydis*.

We showed that after severe oxidative injury, *U. maydis* cell populations restore viability if kept under **s**tarvation conditions (RUS). This restitution of viability is based on cell multiplication at the expense of the intracellular compounds released from the damaged cells. We examined cellular mechanisms involved in reabsorption of the released compounds and in contending with their treatment-induced toxicity. Nine genes contributing to the process were identified by mutant hunt (*adr1, did4, kel1, tbp1, snf8, slm1, chk1, snf5 and hypothetical heat shock UMAG11087).* Mainly from the studies on other organisms, these cellular factors are known to play roles in growth regulation, protein turnover, cytoskeleton organization, transcription, cell cycle regulation, chromatin remodelling and stress response. What is more important, some mutants exhibited sensitivity to different genotoxic agents implying that the gene products are in some overlapping fashion involved in the protection of genome integrity. We also compered RUS activities of *U. maydis* with evolutionary distant model organism *Saccharomyces cerevisiae*.

Keywords: oxidative stress, starvation, liquid holding, genome integrity

INTRODUCTION

Ustilago maydis is a unicellular basidiomycete pathogen of maize and teosinte known for its resistance to large doses of UV or ionizing radiation. Many years ago, the fungus was developed by R. Holliday as a model system for studying DNA repair so that

it has been used for analysis of the genes involved in resistance to radiation for decades (HOLLIDAY, 1965). Its life cycle comprises the saprophytic phase (free-living in soil) and the parasitic phase (mycelial growth in infected plants). In both stages it is exposed to unfavourable conditions. In its saprophytic stage, it must contend with UV-irradiation, oxidative stress, DNA-damaging toxins produced by soil bacteria and other fungi, whereas in its parasitic stage, it is subjected to the immune system of its host, largely to reactive oxygen species as a first line of plant defence against pathogens (KELLER *et al.*, 1998).

The knowledge of the cellular mechanisms underlying ecological success of *U. maydis* is still fragmentary. Nevertheless, it is evident from genetic studies on *U. maydis* that recombinational DNA repair has a central role in radiation resistance since mutation in any of the genes that control homologous recombination results in extreme sensitivity to UV and gamma-rays (FERGUSON *et al.*, 1997; KOJIC *et al.*, 2002, 2003). Although such powerful recombination repair would obviously contribute to the fitness of saprophytic cell populations, recombinational mutants, except dss1, are still capable to invade, infect plant, form galls and generate teliospores (HOLLOMAN *et al.*, 2008). Besides, we should bear in mind that all we know about DNA repair comes from studies of highly proliferative cells under optimal laboratory growth conditions. Such conditions are rarely encountered in nature, where, especially in hostile environment (with limited nutrients), cell division is a less-frequent state for free-living microorganisms. Indeed, we know almost nothing about how this organism might maintain its genome integrity under non-growing (starvation) conditions after strong stress. Arguably, this could have been a prevailing environmental pressure that shaped adaptation of this organism to arid ecosystems of Central America as this is the apparent centre of origin for *U. maydis* and its ancestral teosinte hosts (STUKENBROCK and MCDONALD, 2008). In fact, the dominant hypothesis is that behind the extreme radiation resistance of several organisms lies adaptation to desiccation (GLADYSHEV and MESELSON, 2008; SLADE and RADMAN, 2011).

Challenged to find an approach to study survival of *U. maydis* cell populations in hostile conditions such as exposure to oxidative insult and starvation, we entertained the possibility of using liquid holding (LH) as an assay system that may provide insights into molecular players and cellular mechanisms that underlie the ecological fitness of *U. maydis*. Namely, it was found that if yeast cells, *Saccharomyces cerevisiae*, after exposure to X rays or UV irradiation and prior to plating on solid growth medium, are kept in water for 4 days, the surviving fraction increased as compared to that observed after immediate plating (PATRICK and HAYNES, 1964; PATRICK *et al.*, 1964). This remarkable recovery was found to be the consequence of intracellular repair, but the molecular basis of the phenomenon remained largely unknown. Therefore, we monitored

post-oxidative LH recovery (LHR) of treated *U. maydis* cells held under non-nutrient/ starvation conditions. The analysis has shown that the enhancement of viability was achieved by multiplication of the survivors at the expense of the killed cells. Further investigations revealed the cellular factors involved in the recycling of the intracellular compounds as an ambivalent source of rich but risky nutrients. The insight that was most indicative came from the finding that some of the factors required for regrowth are also involved in the maintenance of genome integrity. We have also shown that *U. maydis* and *S. cerevisiae* share a common way of restitution of viability with LH-namely by reproduction of the survivors yet displaying some interesting differences. The study was extended to another stressor (desiccation) and the results are discussed in the light of their ecological and evolutionary implications.

MATERIAL AND METHODS

Organisms and culture methods: A wild type haploid prototroph *U. maydis* 521 strain and wild type prototroph *S. cerevisiae* ML1131 (haploid) strain were grown in mediums described in detail in MILISAVLJEVIC *et al*., 2020.

Peroxide treatment involving LH is described in detail in MILISAVLJEVIC *et al*., 2018 and MILISAVLJEVIC *et al*., 2020. Briefly, early-stationary phase microbial cell cultures were harvested, washed in water, and resuspended at 2×10^7 cells per ml of 10 mM Fe^{3+}-sodium EDTA. H_2O_2 was added to start the Fenton reaction (generation of Reactive Oxygen Species, ROS) and the suspension held at 30˚C for 10 min. Collected cells were washed twice and plated immediately on an appropriate medium to measure survival or incubated at 30˚C with agitation for 1–3 days before plating to measure recovery. 10 µl of 10-fold dilutions from the initial cell suspension were spotted on solid medium. Plates were incubated for three days at 30˚C for colonies to develop.

Post-desiccation LH assay was described in MILISAVLJEVIC *et al*., 2020. Briefly, washed pellets containing 1×10^8 cells were vacuum desiccated (Speedvac Concentrator, Savant Systems), for 16 h. After rehydration in water, cells were spotted on solid medium or incubated for 2 days and aliquots were withdrawn at 4 h intervals over 2 days and assayed for viability, cellular leakage, and chromosomal DNA degradation.

Time-course leakage analysis: Following peroxide or desiccation treatment *U. maydis* and *S. cerevisiae* cells were resuspended in water and incubated in capped tubes mounted on an oscillating shaker at 30 ˚ C. At various time points, 1 ml aliquots of the suspensions were withdrawn, the cells were pelleted by centrifugation and the supernatants were decanted. To insure that the solutions were cell free, the supernatants were passed through Millipore filters (0.45 µm) and then immediately examined for 260-nm-light-absorbing compounds in a Ultrospec 3300 pro spectrophotometer (Amersham Bioscience).

DNA analysis: Genomic DNA was prepared from cells after peroxide treatment by a modified cetyltrimethylammonium bromide (CTAB) extraction method (DOYLE and DOYLE, 1987) and described in MILISAVLJEVIC *et al*., 2018 and 2020.

RESULTS AND DISCUSSION

Previously, we showed that *U. maydis* possesses remarkable capacity to recover after UV radiation, repeating the LHR phenomenon observed in yeast after X-ray and UV irradiation (MILISAVLJEVIC *et al*., 2018). We extended this investigation to oxidative stress, which is in a way common denominator of all environmental stresses including radiation, and which is also generated endogenously. Therefore, we treated *U. maydis* and *S. cerevisiae* with increasing doses of hydrogen peroxide in the presence of Fe^{3+} ions to generate ROS. There was the expected increased lethal effect with increased doses of peroxide **(**Fig. 1A, left panels).

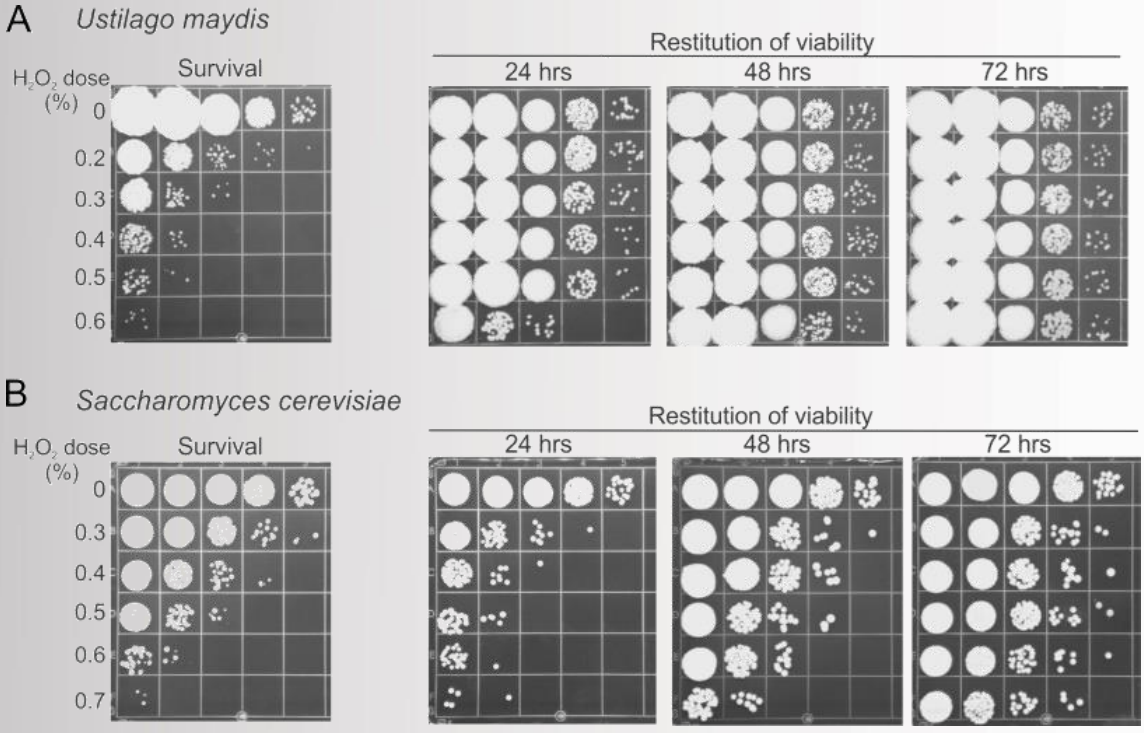

Figure 1. Comparative analysis of the viability of the peroxide treated *U. maydis* (A) and *S. cerevisiae* (B) cells during LH incubation. Aliquots (10 µl) of 10-fold serial dilutions were plated on growth medium for determination of cell viability. Peroxide-treated cells were plated immediately to determine survival or were held in water for the indicated times before plating.

The suspension of *U. maydis* cells exhibited an astonishing degree of restitution of viability during incubation in water. Even when the surviving fraction was approximately 0.001%, the population restitution was almost complete after 72 hour-incubation. Peroxide-treated *S. cerevisiae* cells did also exhibit significant restitution of

viability as a result of post-treatment liquid holding in a non-nutrient medium. Again, the cell suspensions that received the highest doses of peroxide (0.6–0.7%) were capable of increasing the levels of viability by several orders of magnitude over those obtained on the immediate plating. However, the yeast recovery was much slower than that in *U. maydis* and it even showed an initial decline in surviving during the first 24-hour of incubation. After passing through this phase of decline, the viability was progressively increasing as the LH incubation continued, but to the level significantly below that achieved by *U. maydis.*

By several lines of experiments, we showed that the restitution of viability in both microbes is a consequence of multiplication of the undamaged cells at the expense of peroxide killed cells and we named this phenomenon RUS (**R**epopulation **U**nder **S**tarvation conditions). The term "Starvation" indicates here that there was no exogenous nutrient supply. Hence, the survived cells were capable of recycling the oxidatively damaged molecules released from the killed cells (MILISAVLJEVIC *et al*., 2018 and 2020). To investigate the bioavailability of these endogenously derived nutrients, we measured the release of the 260-nm-light-absorbing biomolecules (nucleotidic molecules), as an indicator of the intracellular substances leakage in peroxide treated cell suspensions. The leakage was determined over a 6 h period (or 24 h for *S. cerevisiae*) following the treatment and the time-course curves of the leakage are graphed in Fig. 2A.

The induction of leakage in *U. maydis* was much more drastic; both the rates and extents of the cellular efflux of the nucleotidic compounds in *U. maydis* were much above the equivalents in *S. cerevisiae* (Fig. 2Aa, 2Ac). Next, we examined whether this increase in efflux of nucleotidic compounds involved a concurrent decomposition of the chromosomal DNA. So, the DNA of 0.3% peroxide-treated *U. maydis* cells or 0.5% peroxide-treated yeast cells was extracted and then analysed by agarose gel electrophoresis. As shown, in *U. maydis*, over a period from 0 to 12 h there was an early and dramatic degradation of the DNA followed by the appearance of high molecular weight genomic DNA as the cell population was recovering. (Fig. 2Ab). Nothing remotely similar was observed in *S. cerevisiae* (Fig. 2Ad). The DNA persisted in a similar shape over the entire period of 24 h. Even after prolonged incubation (48 h) no comparably extensive DNA degradation was evidenced.

Since the peroxide treatment might be considered to be too drastic, we have chosen to test generalizability of the observations obtained upon peroxide treatment by employing one of the major environmental stressors, desiccation. By a conceptually similar strategy, we monitored the time course of cell viability, measured the time course leakage of the A_{260} material and performed a time course analysis of the chromosomal

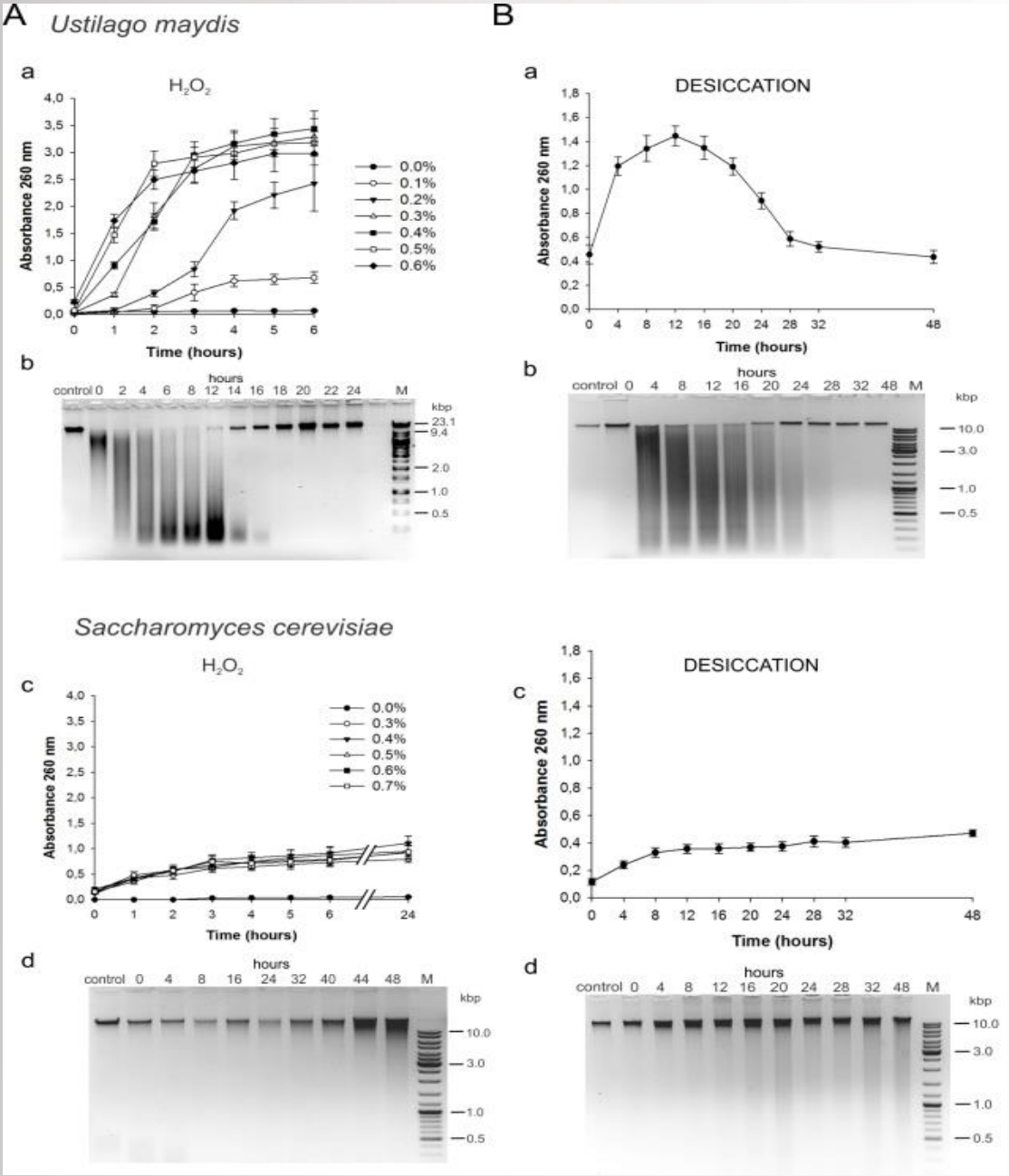

Figure 2. Leakage and decomposition of cellular macromolecules during LH incubation. (A). Time course of leakage of presumptive nucleotidic material from H_2O_2-treated cells of *U. maydis* (Aa) and *S. cerevisiae* (Ac). 2×10^7 wild type cells per ml were treated with indicated doses of peroxide, washed and incubated in water for 6 h (*U. maydis*) or 24 h (*S. cerevisiae).* Aliquots were taken, centrifuged and millipore-filtered supernatants were used for measurements of absorbance at 260 nm periodically. The error bars indicate standard deviations of three independent experiments. Genomic DNA was analyzed at indicated time points after cells were peroxide-treated: *U. maydis* (0.3% H_2O_2) (Ab) and *S. cerevisiae* (0.5% H_2O_2) (Ad) and then incubated in water for 24 or 48 h, respectively. The control shows DNA from untreated cells. M: Mix of GeneRuler DNA Ladder Mix and Lambda DNA/HindIII Marker (Thermo Scientific). (B) Restitution of viability of desiccated *U. maydis* and *S. cerevisiae* cells during LH incubation. Leakage of molecules (Ba, Bc) was monitored as in A; Extracted genomic DNA at the indicated time points was analysed by agarose gel electrophoresis. (Bb, Bd).

DNA degradation in *U. maydis* and *S. cerevisiae* cell suspensions recovering from effects of desiccation. The results are shown in Fig. 2B. The same general patterns of *U. maydis* and *S. cerevisiae* post-stress response were clearly observed: a rapid leakage of the cytosol compounds from the killed cells and rapid degradation of the chromosomal DNA in *U. maydis* (Fig. 2Ba, 2Bb) and a low amount of bioavailable compounds released in the yeast suspensions, coincidental with no substantial degradation of the genomic DNA (Fig. 2Bc, 2Bd).

Thus, together, these results show that the evidence obtained from peroxide treatment is extendable to another stressor (desiccation), suggesting its reliability and significance from an ecological point of view.

Faster leakage of biomolecules certainly contributes to the superior RUS performance of *U. maydis*. However, the ability to effectively exploit the freed biomolecules could also contribute to the *U. maydis*' efficacy. To relate *U. maydis* and *S. cerevisiae* in this regard we compared their growth in supernatants derived from *U. maydis* cultures treated with peroxide (Fig. 3). Therefore, suspensions of *U. maydis* cells were treated with increasing doses of hydrogen peroxide (between 0 and 1.1%), the killing was measured by ability to form colonies on immediate plating, and after being washed two times the suspensions were incubated in water for 4 hours. The cell-free supernatants were than inoculated with either *U. maydis* or *S. cerevisiae* fresh cells ($8x10^3$). Incubation was carried out as above and the growth was examined at 1-day intervals.

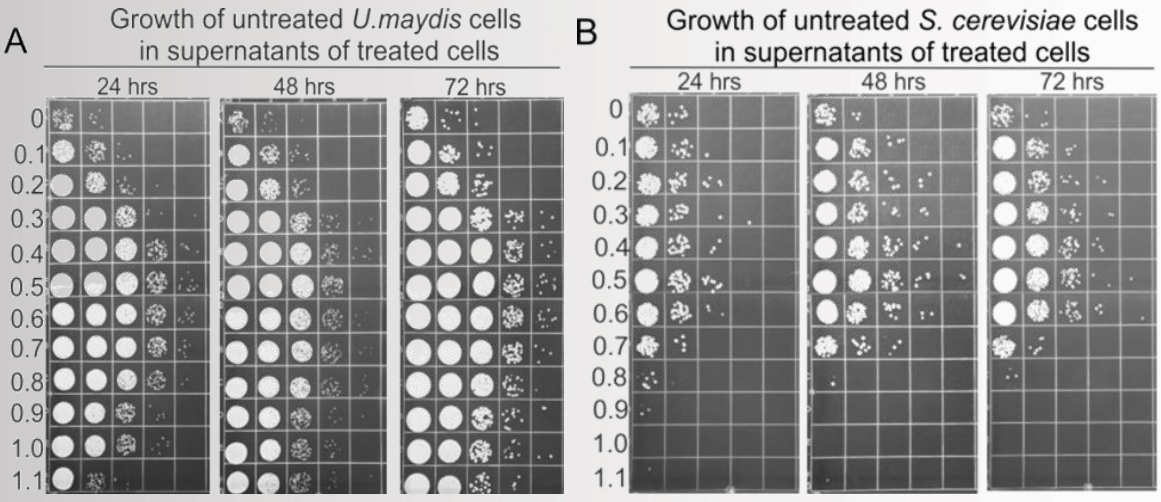

Figure 3. Cell growth effected by the substrates released from peroxide treated cells. (A) Growth of untreated *U. maydis* and (B) *S. cerevisiae* cells in cell-free supernatants derived from *U. maydis* cells treated with indicated doses of peroxide. 2×10^7 cells were treated, washed and incubated in water for 4 h. Cell-free supernatants obtained by centrifugation and millipore-filtration were inoculated with 8×10^3 fresh cells and incubated for 3 days in water at 30 C under continuous agitation before serial dilution and plating on the complete medium. Pictures are representative of at least two independent experiments.

On the whole, *U. maydis* exhibited faster and more prolific growth. *S. cerevisiae* was not only lagging behind *U. maydis* across the entire spectrum of supernatants but was also totally inhibited in growth when inoculated in the highest dosages *U. maydis* supernatants (from 0.6−0.8%) and even dying off in the supernatant derived from 0.8 -1.1% peroxide-treated cells.

In conclusion, *U. maydis* proved itself to be superior to *S. cerevisiae* in every aspect of exploitation of the nutritional resources derived from peroxide treated cells. The most striking difference was revealed under severe challenge suggesting that *U. maydis* cells are much more competent for handling and utilization of potentially harmful cellular waste. Therefore, we conclude that the reason for more efficient RUS in *U. maydis* is twofold: (1) greater bioavailability of nutritional resources and (2) greater ability of *U. maydis* cells to recycle damaged and released intracellular compounds. Accordingly, these features may contribute to a superior survival in hostile environments that are frequently encountered in the wild.

To get insight into the cellular processes required for recycling of the oxidativley damaged biomolecules, we isolated *U. maydis* RUS mutants incapable to reuse this sort of nutrients (MILISAVLJEVIC *et al*., 2018). The isolated factors have quite diverse cellular functions: growth regulation (Adr1), vesicular transport (Snf8, Did4, Vps54), actin organisation (Kel1, Slm1), transcription regulation (Mot2, Tbp1), chromatin remodelling (Snf5) and stress response (hypothetical heat shock UMAG11087). Importantly, some of the mutants were also incapable of recovering after desiccation, implying that these two processes share at least some of the components of cellular machinery (MILISAVLJEVIC *et al*., 2020). Moreover, the mutants were tested for sensitivity to various genotoxin agents introducing different types of DNA lesions: UV, MMS (methyl methanesulfonate) and HU (hydroxyurea). We found that some of the mutants showed sensitivity to genotoxins to a different extent (Fig. 4).

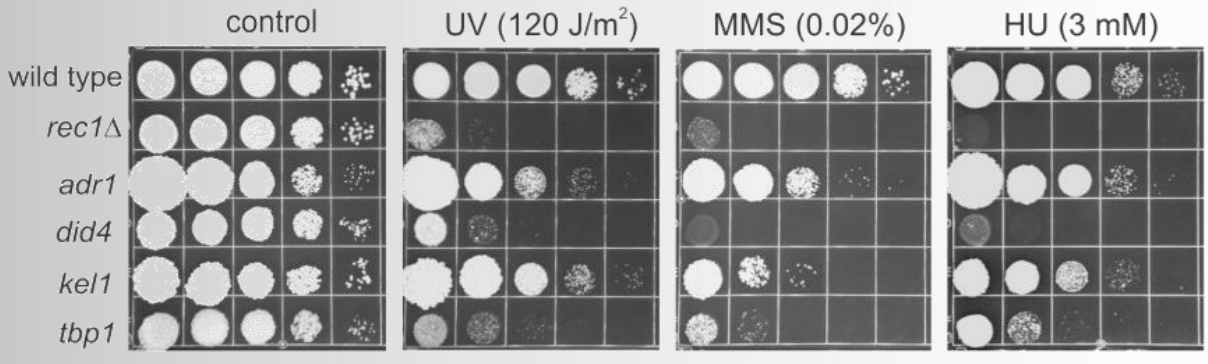

Figure 4. Response of RUS mutants to genotoxins. Mutants were plated on solid medium then irradiated with UV or else plated on medium containing MMS or HU. The rec1Δ mutant was used as a control to illustrate sensitivity to all three treatments. The testing was performed two times and representative results are shown.

These finding are of special note because they are most suggestive of the possibility that at least some of the cellular factors involved in the management of the leaked intracellular compounds caused by peroxide and desiccation also do operate in some principal and overlapping manner in the response to DNA damage and replication stress. In addition, this implies that RUS can be seen as a valuable tool for identifying new cellular factors dedicated to the maintenance of genome integrity.

CONCLUSION

Overall, we showed that two evolutionary distant microbes are both capable of performing RUS after at least two stresses: peroxide-imposed oxidative stress and desiccation. The different RUS capacities observed in *U. maydis* and *S. cerevisiae* may reflect specific adaptations of these fungi to diverse demands of their ecological niches. RUS can be seen as a post-stress activation of complex cellular mechanisms that enables renewal of population of microorganisms in the aftermath of devastating environmental stresses. Populations are not uniformly affected by severe stresses so that the survivors could re-establish the population at the expense of the dead biomass. In *U. maydis* the superiority in recycling of the damaged and released compounds may, in inhospitable environments, provide a major input to the ecological and evolutional advantage over other microorganisms as well as even contribute to the extreme radiation resistance of this remarkable fungus.

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REFERENCES

Doyle J.J., Doyle J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull., 19, 11-15.

Ferguson D.O., Rice M.C., Rendi M.H., Kotani H., Kmiec E.B., Holloman W.K. (1997). Interaction between *Ustilago maydis* REC2 and RAD51 genes in DNA repair and mitotic recombination. Genetics, 145, 243–251.

Gladyshev E., Meselson M. (2008). Extreme resistance of bdelloid rotifers to ionizing radiation. Proc. Natl. Acad. Sci. U.S.A. 105, 5139–5144.

Holliday R. (1965). Radiation sensitive mutants of *Ustilago maydis*. Mutat. Res. 2, 557–559.

Holloman W.K., Schirawski J., Holliday R. (2008). The homologous recombination system of *Ustilago maydis*. Fungal Genet. Boil. 45(Suppl. 1), S31–S39

Keller T., Damude H. G., Werner D., Doerner P., Dixon R.A., Lamb C. (1998). A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca2+ binding motifs. Plant Cell 10, 255–266.

REML ANALYSIS OF RANDOM SEGMENT OF THE MODEL FOR GROWTH AND CARCASS VALUE TRAITS IN GILTS

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ABSTRACT

The aim of this paper was to assess the ratio of random effects in total phenotypic variability of growth and carcass value traits in gilts in order to obtain necessary parameters for assessing the genetic potential of these animals by means of some reference methods (selection index, BLAP-AM). Following gilt traits were analysed: daily live-weight gain at the end of the test (ADG), age at the body mass of 100 kg (TT), average loins and back fat thickness (BF), *longissimus dorsi* muscle depth (DM) and percentage of meat in carcass (PM). All examined traits were analysed for two random effects, i.e. the litter in which gilts were born and raised and an animal direct additive genetic effect estimated on the basis of kinship matrix. It was determined that litter in which gilts were born and raised accounts for 45% of total variability for ADG, 13% for TT, 6% for BF, 6% for MLD and 7% PM. An animal direct additive genetic effect, that is, heritability coefficients for ADG, TT, BF, DM and PM for all traits were h^2 = 0.6; 0.24; 0.52; 0.6; 51, respectively.

Keywords: gilts, heritability, mixed modesl, random effect, REML- analysis

INTRODUCTION

Production in pig breeding directly depends on all economically important swine trait groups. Over previous decades in pig populations in the Republic of Serbia a considerable advancement was made regarding genetic improvement in growth trait, feed conversion and carcass quality in pigs.

One of the main preconditions during the process of genetic improvement of traits in any species of domestic animals is to estimate, as precisely as possible, heritability of traits, on the basis of which the selection is being conducted, i.e. to differentiate between genetic and non-genetic effects in the expression of some trait so that by use of one of reference methods (BLUP (AM) - Best Linear Unbiased Prediction

(Animal Model), method of selection indices) a genetic potential of an animal for a specific type of production could be perceived. Owing to a relatively high heritability of growth trait and carcass quality trait in pigs and possible use of simpler methods of selection in improving these traits a significant genetic progress has been made therein.

Recognizing different factors of variability and specificity of their action is one of the key factors of accuracy of obtained scores of genetic components of traits variances, necessary for their improving. A generally accepted practice is to use REML – method (Restricted Maximum Likelihood - method of restricted maximum likelihood) in the traits characterized by a normal distribution (growth traits and carcass quality, some reproductive traits, etc.) along with the use of methodology of mixed models. Manifestation of this group of traits is affected by a great number of non-genetic and genetic factors which are relatively simple to identify and determine in comparison with the factors which affect some other groups of traits (fertility traits), what all resulted in the fact that growth trait and carcass traits in pigs in the last thirty years accomplished high genetic improvement compared to other traits. Moreover, intensive selection, application of simpler methodological procedures of selection (method of selection indices) and medium heritability of this group of traits with heritability coefficients ranging from 0.2 to 0.5 made the highest genetic progress to be attained in this group of traits. Since in this research paper the emphasis is on the analysis of random effects, i.e. litters in which gilts were born and raised and on animal direct additive effect, on variability of this group of traits, literature data shall also be based on these factors of variability of the growth traits and carcass quality in performance tested gilts. A practical application of methodology of mixed models (MMM - Mixed Model Methodology) in the last two decades in selection of domestic animals, which implied the use of kinship matrix, has enabled precise determination of ***direct additive genetic effect*** in entire phenotypic variance of some trait included into a certain selection programme. In literature this effect is often called the effect of an individual and it represents the sum of effects of individual alleles of a large number of genes which control manifestation of a certain trait. Each parent passes onto its offspring half of its own hereditary basis what is, on the basis of their own performance and on the performance of their relatives and by respecting other determined effects, a sufficient material basis to precisely estimate variance additive genetic component which being expressed in relation to total phenotypic variance, represents a heritability coefficient (heritability) of some trait (RADOJKOVIĆ, 2007).

In order to obtain more precise estimation of genetic parameters when the traits of growth and carcass quality are in question, today has become a rule to include the ***litter in which gilt was born and raised*** as a random effect in the models on the basis of which these parameters are estimated. This effect implies combining of all those effects

which dominated before the birth of some litter – gilts (intrauterine period), as well as those effects which dominated after the birth of an animal all through to its weaning or even up to the end of its raising as a piglet if the piglets from the same litter are raised in the same group, i.e. in the same cage or box. It is thought that litter in which gilts were born and raised leads to an increased similarity between animals from the same litter, i.e. the difference between the animals which originate from different litters is increasing. This effect involves a whole range of effects such as: nutrition, environment conditions, litter health state, maternal characteristics of dam of litter, social hierarchy in a litter and all those effects which are specified for every litter and are not described by some other factor of variability of studied trait (POPOVAC, 2016).

Table 1. shows data on relative proportion of random effects in total variability of traits of growth and carcass quality in performance tested pigs obtained in different research studies.

On the basis of different literature data on the proportion of random effects in variability of traits of growth and carcass quality in gilts shown in Table 1, it can be said that majority of studies indicates medium heritability of this group of traits with a smaller number of deviations while a proportion of random effect of litter explains from 0 to 20% of variability of these traits in the majority of studies mentioned.

MATERIAL AND METHOD

The trials were conducted on a pig farm in the Republic of Serbia. The research included gilts of two genotypes such as: purebred Swedish Landrace animals and crossbreds of F1 generation of Swedish Landrace and Great Yorkshire wherein a maternal breed in this combination of cross-breeding were Swedish Landrace breeding females.

The aim of the research was to study variability and heritability of traits in performance test of gilts in order to discern a possibility for potential estimation of breeding value of these animals by BLUP – AM method or by method of selection indices.

A data set containing complete records for 4768 gilts born and producing in the 17-year period was formed. Out of total number of gilts whose traits were analysed, 2876 animals were of Swedish Landrace breed and 1892 animals were crossbreds of F1 generation.

Analysed traits, units of measure in which they are expressed and their abbreviations are presented in Table 2 while the way of defining of all the traits is shown in Table 3.

Table 1. Data on relative proportion of random effects in total variability of traits of growth and carcass quality in performance tested pigs

Research study	DG		TT		SL		DM		PM	
	h^2	l^2	h^2	l^2	h^2	l^2	h^2	l^2	h^2	l^2
Brkić (1998)	0.25				0.78		0.08		0.75	
Malovrh and Kovač (1999)	0.14 0.16	0.22			0.35 0.11	0.06 0.15				
Bizelis et al. (2000)			0.60 0.36		0.44		0.51 0.37			
Crump (2001)	0.23	0.10			0.50	0.04				
Brkić (2002)	0.11		0.10		0.46 0.64		0.11		0.63	
Vuković (2003)	0.28 0.36		0.30 0.13		0.45					
Vincek et al. (2003)			0.00 – 0.35	0.06 – 0.0	0.01 – 0.30	0.07 – 0.19				
Serenius and Stalder (2004)	0.40				0.32 0.32					
Solanes et al. (2004)	0.43				0.53 0.64	0.03 0.05			0.67	
Arango et al. (2005)	0.15									
Nguyen and McPhee (2005)	0.19	0.17			0.25	0.05			0.40	0.00
Suzuki et al. (2005)					0.72	0.01				
Imboonta et al. (2007a)	0.38				0.61					
Imboonta et al. (2007b)	0.31				0.45					
Kasprzyk (2007)	0.28 0.46				0.12 0.15		0.12		0.59 0.17	
Vukovic et al. (2007)	0.27				0.38					
Cai et al. (2008)	0.42	0.00			0.68	0.08				
Nagy et al. (2008)	0.20	0.29 – 0.03	0.23	0.46 – 0.48					0.23 0.28	0.20 0.25
Jones et al. (2009)	0.31 0.22	0.08 0.12			0.68	0.02 0.03				
Szyndler – Nędza (2010)	0.30 0.39				0.14 0.15 0.17		0.05 0.16		0.10 0.19	
Wolf and Wolfová (2012)	0.12 0.20	0.14 0.22							0.32 0.38	0.08 0.09
Popovac et al. (2014a)	0.26				0.36				0.36	
Popovac et al. (2014b)	0.38				0.26					
Wongsakajornkit and Imboonta (2015)	0.31 0.23				0.57 0.46					

h^2 – heritability estimation (heritability coefficient); l^2 – estimation of the effect of litter in which gilts were born/raised;

Table 2. Analysed traits, units of measure and abbreviations of traits

GROUP OF TRAITS	TRAIT	UNITS OF MEASURE	ABBREV.
GROWTH TRAITS	Live weight gain	Gram	**ADG**
	Age at the end of test	Day	**TT**
CARCASS QUALITY TRAITS	Fat thickness	Millimeter	**BF**
	Depth of DM	Millimeter	**DM**
	Carcass meat percentage	Percentage	**PM**

Table 3. Defining of studied traits

Trait	TRAITS DEFINING
ADG	(Body mass at the end of test gilts – 1.25 kg) / Age at the end of test gilts
TT	Number of days from birth to the end of performance test gilts, corrected for the mass of 100 kg
BF	(Loins fat thickness + back fat thickness) / 2
DM	Depth of DM – measured in loins
PM	Percentage of meat determined by regression equation on the basis of thickness of fat in loins and in back and depth of DM at the end of performance test

As the research programme envisaged the study of an animal additive effect in evaluation of genetic parameters of studied traits the next step was to construct a pedigree file necessary for the realisation of such set research goal. Pedigree file is used to form a kinship matrix which serves to determine a precise relations (kinship) between animals whose performance was analysed as well as performance of their ancestors and lateral relatives all with the aim of estimating genetic parameters in studied pig population as precise as possible. Data checking in pedigree file and determining of total number of animals in the file was perfomed by means of Pedigree Viewer 6.5 programme package (KINGHORN, 1994). Structure of data and pedigree file on whose basis the research was conducted is shown in Table 4.

Table 4. Structure of data and pedigree file used in analysis

PARAMETER	Number	Parameter	Number
NUMBER OF GILTS	4768	Number of base animals in pedigree file (unknown origin)	287
TOTAL NUMBER OF ANIMALS IN PEDIGREE FILE	5666	Percent of base animals in pedigree file (%)	5.07
NUMBER OF ANIMALS WITH PRODUCTION DATA IN PEDIGREE FILE	4768	Average coefficient of inbreeding	0.145
NUMBER OF ANCESTORS IN PEDIGREE FILE	898		

The process of styding the variability of the traits by linear methods was performed by a *step by step* principle which meant first the development and analysis of the so-called systematic component of the model composed of fixed and regression effects. By the least squares method and use of GLM procedure with help of ”SAS/STAT“ (SAS Inst. Inc., 2010) programme package**,** systematic effects were examined and on that basis the choice of animals that **showed a statistically significant effect on trait variability and were on the basis of that involved in the final** (mixed) model was performed and on that basis the values of genetic parameteres of studied traits were estimated.

In the analysis of fixed part of model for the traits in gilt performance test (ADG, BF, DM, PM), we started from the following model of the method of the least squares while the variability of TT was studied on the basis of model 2, because a previous correction of this trait to the body mass of 100 kg was done.

Model 1.

$$Y_{ijk} = \mu + G_i + S_j + b_1 (x_{ijk} - \bar{x}) + e_{ijk}$$

Model 2.

$$Y_{ijk} = \mu + G_i + S_j + e_{ijk}$$

where:

Y_{ijk} – is manifestation of studied trait of k individual, μ – general average of studied trait in population, G_i – fixed effect of genotype, S_j – fixed effect of season at the end of performance test defined by year/month interaction, $b_1 (x_{ijk} - \bar{x})$ – linear regression effect of body mass measured at the end of test, e_{ijk} – effect of non-determined factors (the rest).

The next step in the process of analysis was, on the basis of mixed models, to estimate variances of studied traits as well as the ratio of some variances in total phenotypic variance all with the aim of establishing heritability of these traits. Construction of mixed models was performed in such a way that they contained all statistically significant effects determined by previous analysis of systematic components of model and random effects whose inclusion was biologically justified. Since the research was conducted in pig population in which selection was performed over a long period in different ways and the sample of selected sows was not random, for the estimation of variances and covariances the method of restricted maximum likelihood was used (REML – Restricted Maximum Likelihood) by means of a single trait model (ST – Single Trait).

Estimation of variances was performed in ”VCE – 6“ programme package (GROENEVELD *et al*., 2010), while the preparation of data for analysis in previously mentioned software which implied so-called coding of data, was done by help of ”PEST“ programme package (GROENEVELD *et al*., 1990).

For the evaluation of variances in ADG, BF, PM necessary for calculating the heritability and correlation of studied traits model 3 was used, for TT model 4 and for DM model 5.

Model 3.

$$Y_{ijkl} = \mu + G_i + S_j + b_1 (x_{ijkl} - \bar{x}) + l_k + a_{ijkl} + e_{ijkl}$$

Model 4.

$$Y_{ijk} = \mu + S_i + b_1 (x_{ijk} - \bar{x}) + l_j + a_{ijk} + e_{ijk}$$

Model 5.

$$Y_{ijkl} = \mu + G_i + S_j + l_k + a_{ijkl} + e_{ijkl}$$

where:

Y_{ijkl} – is manifestation of studied trait of l individual, μ – general average of studied trait in population, G_i – fixed effect of genotype, S_j – fixed effect of season at the end of performance test defined by year/month interaction, $b_1 (x_{ijk} - \bar{x})$ – linear regression effect of body mass at the end of test, l_k – random effect of litter in which gilts were born/raised, a_{ijkl} – animal direct additive genetic effect (breeding value), e_{ijkl} – effect of non-determined factors (the rest).

Heritability coefficient (heritability) of analysed traits is presented as a relationship of additive genetic variance determined by REML method by means of kinship matrix and total phenotypic variance what can be presented by a following expression:

$$h^2 = \frac{\sigma_a^2}{\sigma_{ph}^2}$$

where:

h^2 – is a heritability coefficient (heritability),

σ_a^2 – additive genetic variance,

σ_{ph}^2 – total phenotypic variance.

RESULTS AND DISCUSSION

Table 5 shows average values and variation measures (descriptive statistical parameters) for studied traits of growth and gilt carcass quality determined at the end of performance test.

Table 5. Descriptive statistical parameters for traits of growth and carcass quality in gilts

Trait	N	$\bar{X}$	SD	Min	Max	CV
ADG (g/day)	4768	525.51	43.51	367	667	8.28
TT (day)	4768	189.53	15.77	149	269	8.32
BF (mm)	4768	14.50	3.58	7	30	24.68
DM (mm)	4768	47.43	4.29	35	69	9.05
PM (%)	4768	56.4	3.53	41.6	64.7	6.26

Average values of analysed traits were as follows: ADG – 525.51g/day; TT – 189.53days; BF – 14.50mm; DM – 47.43mm; PM – 56.44%.

Table 6 shows singular relative ratio of random effects in total phenotypic variance of the traits of growth and carcass quality in performance tested gilts *(model with one trait).*

Table 6. Relative ratio of random effects in total phenotypic variance of the traits of growth and carcass quality in performance tested gilts

Trait	$h^2 \pm se$ (h^2)	$l^2 \pm se$ (l^2)	$e^2 \pm se$ (e^2)
ADG	**0.06** ± 0.03	0.45 ± 0.03	0.49 ± 0.03
TT	**0.24** ± 0.03	0.13 ± 0.02	0.63 ± 0.04
BF	**0.52** ± 0.04	0.06 ± 0.02	0.41 ± 0.04
DM	**0.06** ± 0.02	0.06 ± 0.03	0.88 ± 0.03
PM	**0.51** ± 0.04	0.07 ± 0.02	0.42 ± 0.04

h^2 – heritability estimation (heritability coefficient); l^2 – estimation of the effect of litter in which gilts were born/raised; e^2 – error estimation (the rest)

Heritability estimation for ADG of 6 % indicates a very low heritability of this trait in examined pig population and this value is significantly lower compared to all the values found in available literature regardless the method used for heritability estimation (BRKIĆ, 1998 and 2002; MALOVRH and KOVAČ, 1999; CRUMP, 2001; VUKOVIĆ, 2003; SERENIUS and STALDER, 2004; SOLANES *et al*., 2004; ARANGO *et al*., 2005; NGUYEN and MCPHEE, 2005; IMBOONTA *et al*., 2007a and 2007b; KASPRZYK, 2007; VUKOVIC

et al., 2007; CAI *et al*., 2008; NAGY *et al*., 2008; JONES *et al*., 2009; SZYNDLER – NĘDZA *et al*., 2010; WOLF and WOLFOVÁ, 2012; POPOVAC *et al*., 2014a and 2014b; WONGSAKAJORNKIT and IMBOONTA, 2015). In addition, presented value of heritability deviate from generally accepted standpoint confirmed by majority of previously mentioned studies that this trait is characterized by a medium heritability where heritability coefficient values are in the interval of 0.2 to 0.5.

On the other hand, a relatively high percentage of litter in which gilts were born and raised of 0.49 in total phenotypic variance of live weight gain is significantly higher compared to a relative percentage of this variance component determined in the studies by MALOVRH and KOVAČ (1999), CRUMP (2001), NGUYEN and MCPHEE (2005), CAI *et al*. (2008), NAGY *et al*. (2008), JONES *et al*. (2009) and WOLF and WOLFOVÁ (2012).

In the previous table we can see that direct additive genetic effect explains 0.24 of total variability of TT in studied gilts. Obtained value of heritability for this trait is within the range of values which in their research obtained VINCEK *et al*. (2003), VUKOVIĆ (2003) and NAGY *et al*. (2008), while it is higher compared to the value reported by BRKIĆ (2002), and lower compared to the value stated by BIZELIS *et al*. (2000).

The litter in which gilts were born and raised explains 0.13 of total TT variance. Comparing the value obtained for this effect with the values available in literature it can be seen that is within the frame of the values determined by VINCEK *et al*. (2003), who explained from 6 to 0.40 of total variance of this trait by this factor while it is significantly lower compared to the values reported by NAGY *et al*. (2008).

Heritability coefficient of 0.52 determined for BF of gilts shows that this trait in studied population of pigs is in the category of high heritability traits what is the value similar to those (±0.10) determined in the research studies of BIZELIS *et al*. (2000), CRUMP (2001), BRKIĆ (2002), VUKOVIĆ (2003), SOLANES *et al*. (2004), IMBOONTA *et al*. (2007a and 2007b) and WONGSAKAJORNKIT and IMBOONTA (2015). On the other hand, displayed value of heritability in this research for DS is smaller in relation to the values reported by BRKIĆ (1998), SUZUKI *et al*. (2005) and CAI *et al*. (2008), while it is higher in relation to the values obtained by MALOVRH and KOVAČ (1999), VINCEK *et al*. (2003), SERENIUS and STALDER (2004), NGUYEN and MCPHEE (2005), KASPRZYK (2007), VUKOVIC *et al*. (2007), JONES et al. (2009), SZYNDLER – NĘDZA et al. (2010) and POPOVAC *et al*. (2014a and 2014b), which were in the range of 1 to 0.38.

Gilt birth litter had a several times less ratio (0.06) in relation to genetic additive component of variance for BF in total phenotypic variance of this trait. Relative percentage of this effect in total variance of studied trait determined in this research is similar to the percentage (<10 %) determined by CRUMP (2001), SOLANES *et al*. (2004), NGUYEN and MCPHEE (2005), SUZUKI *et al*. (2005), CAI *et al*. (2008), JONES *et al*. (2009)

and to the part of the results regarding relative value of this variance component in total variance determined by MALOVRH and KOVAČ (1999) and VINCEK *et al*. (2003), taking into consideration that the last two groups of authors determined this variance component in some pig populations to account for 0.15 i.e. 0.19.

As for heritability regarding DM, only 6% of total variability of this trait was explained by a direct genetic additive effect what is deemed a very low value of heritability if we take into account that the traits of carcass quality are generally characterised by a medium to high heritability. On the other hand, obtained value of heritability is confirmed by the results obtained by: BRKIĆ (1998 and 2002), KASPRZYK (2007) and SZYNDLER – NĘDZA *et al*. (2010), who also obtained low heritability of this trait. The results regarding heritability of DM are inconsistent only with the results presented by BIZELIS *et al*. (2000), who determined medium, i.e. high heritability of this trait.

The litter in which gilts were born and raised, as well as a direct additive effect, accounts for 0.06 of total phenotypic variance of DM in gilts.

PM variability in the carcass of performance tested gilts was conditioned by 0.51 of the animal direct additive genetic effect. A heritability value determined indicates a high heritability of this trait what agrees with the research results obtained by BRKIĆ (1998 and 2002), SOLANES *et al*. (2004) and with a part of the results of KASPRZYK (2007), the value of heritability for PM obtained in this research being lower compared to the values determined by previously mentioned groups of authors. On the other hand, determined heritability of this trait disagree with the results of NGUYEN and McPHEE (2005), NAGY *et al*. (2008), WOLF and WOLFOVÁ (2012) and POPOVAC *et al*. (2014a) who determined a medium heritability of this trait and with the results of SZYNDLER – NĘDZA *et al*. (2010) and part of the results reported by KASPRZYK (2007), who determined a low heritability of PM in the carcass of performance tested pigs.

Total phenotypic variability of carcass PM was conditioned by 0.07 of litter in which gilts were born and raised. It is a similar relative value of this variance component to the values determined by WOLF and WOLFOVÁ (2012), which deviate from the values reported by NGUYEN and McPHEE (2005) who found this variance component to be close to zero and to the values reported by NAGY *et al*. (2008), who explained 0.20, i.e. 0.25 variability of this trait by this random factor.

CONCLUSION

The values of heritability coefficients for TT, BF and PM of h^2= 0.24; 0.52; 0.51, indicate medium, i.e. high heritability of these traits what was, besides this research, determined in a number of other research studies as well. On the other hand, the value of h^2= 0.06 determined for ADG and DM in this research classify these traits into a group of

low heritability traits what diverge from a general standpoint that these traits have medium, i.e. high heritability.

The percentage of litter in which gilts were born and raised significantly contributed to a better explanation of total variability of traits and to more precise estimation of variance genetic components, thus by this random effect following percentages of variability: ADG – 0.45, TT – 0.13, BF – 0.06, DM – 0.06, PM – 0.07 were explained.

The issue which should certainly be paid attention to is why in the traits in the same group which should be highly correlated (for example ADG and TT), the values explained by a variability of the same random effects can differ significantly.

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REFERENCES

Arango J., Misztal I., Tsuruta S., Culbertson M., Herring W. (2005): Estimation of variance components including competitive effects of Large White growing gilts. Journal of Animal Science, 83, (6), 1241-1246.

Bizelis J., Kominakis A., Rogdakis E., Georgadopoulou F. (2000): Genetic parameters of production and reproduetive traits in on a farm tested Danish Large White and Landrace swine in Greece. Archiv Tierzucht, 43, (3), 287-297.

Brkić N. (1998): Ocena priplodne vrednosti performans testiranih nazimica primenom selekcijskih indeksa. Magistarska teza. Univerzitet u Beogradu, Poljoprivredni fakultet Beograd, 1-121 str.

Brkić N. (2002): Ocena odgajivačke vrednosti priplodnih grla svinja primenom integrisanog selekcijskog indeksa. Doktorska disertacija. Univerzitet u Beogradu, Poljoprivredni fakultet Beograd, 1-165 str.

Cai W., Casey D., Dekkers J. (2008): Selection response and genetic parameters for residual feed intakein Yorkshire swine. Journal of Animal Science, 86, (2), 287–298.

Crump E. (2001): A genetic analysis of sow longevity. In: Proceedings of the 14th Conference of the Association for the Advancement of Animal Breeding and Genetics, New Zealand, Queenstown, September 2001., vol. 14, 223–226 pp.

Groeneveld E., Kovač M., Wang T. (1990): PEST, a general purpose BLUP package for multivariate prediction and estimation. In: 4th World Congress on Genetics Applied to Livestock Production, Skotland, Edinburgh, Jun 1990., vol. 13, 488-491 pp.

Groeneveld E., Kovac M., Mielenz N. (2010): VCE6 User's Guide and Reference Manual.Mariensee, Institute of Farm Animal Genetics, FLI: 125 pp.

Imboonta N., Rydhmer L., Tumwasorn S., (2007a): Genetic parameters for reproduction and production traits of Landrace sows in Thailand. Journal of Animal Science, 85, (1), 53-59.

Imboonta N., Rydhmer L., Tumwasorn S. (2007b): Genetic parameters and trends for production and reproduction traits in Thai Landrace sows. Livestock Science, 111, (1), 70–79.

Jones R., Hermesch S., Crump R. (2009): Evaluation of pig flight time, average daily gain and backfat using random effect models including grower group. In: Proceedings of the 18th Conference of the Association for the Advancement of Animal Breeding and Genetics, September 2009., vol. 18, 199-202 pp.

Kasprzyk A. (2007): Characteristics of genetic parameters and genetic gain in breeding herd of PL pigs over 25- year breeding work period. Archiv Tierzucht, 50, (Special Issue), 107-115.

Kinghorn, B.P. (1994): Pedigree Viewer - a graphical utility for browsing pedigreed data sets. 5th World Congress on Genetics Applied to Livestock Production. Guelph, 7 - 12 August 1994. 22: 85-86.

Malovrh Š., Kovač M. (1999): Genetic correlations between test station and on-farm performance for backfat thickness and daily gain. Acta Agraria Kaposváriensis, 3, (2), 145-154.

Nagy I., Csató I., Farkas J., Gyovai P., Radnóczi L., Komlósi I. (2008): Genetic parameters of direct and ratio traits from field and station tests of pigs. Archiv Tierzucht, 51, (2), 172-178.

Nguyen N., McPhee C. (2005): Genetic parameters and responses of performance and body composition traits in pigs selected for high and low growth rate on a fixed ration over a set time. Genetics Selection Evolution, 37, (2), 199–213.

Popovac M. (2016): Fenotypic and genetic variability of sows productive and longevity traits. Doctoral dissertation, University of Belgrade, Faculty of Agriculture, 1-178 pp.

Popovac M., Petrović M., Radojković D., Stanojević D., Miletić A., Perišić P. (2014a): The assessment of genetic potential in performance tested gilts by means of selection indexes method. Genetika, 46, (1), 95-104.

Popovac M., Radojković D., Petrović M., Gogić M., Savić R., Stanojević D., Miletić A., (2014b): The assessment of breeding value of first farrowed sows by the method selection indices. In: Proceedings of the international symposium on animal science 2014, Serbia, Belgrade, September 2014, 135-146 pp.

Radojković D. (2007): Varijabilnost osobina plodnosti i procena priplodne vrednosti svinja. Doktorska disertacija. Univerzitet u Beogradu, Poljoprivredni fakultet Beograd, 1-208 str.

SAS Inst. Inc. (2010): SAS/STAT® 9.3_M1 User's Guide. Cary, NC

Serenius T., Stalder K. (2004): Genetics of length of productive life and lifetime prolificacy in the Finnish Landrace and Large White pig populations. Journal of Animal Science, 82, (13), 3111–3117.

Solanes F., Grandinson K., Rydhmer L., Stern S., Andersson K., Lundeheim N. (2004): Direct and maternal influences on the early growth, fattening performance, and carcass traits of pigs. Livestock Production Science, 88, (3), 199–212.

Suzuki K., Kadowaki H., Shibata T., Uchida H., Nishida A. (2005): Selection for daily gain, loin-eye area, backfat thickness and intramuscular fat based on desired gains over seven generations of Duroc pigs. Livestock Production Science, 97, (2-3), 193–202.

Szyndler-Nędza M., Tyra M., Różycki M. (2010): Coefficients of heritability for fattening and slaughter traits included in a modified performance testing method. Ann. Anim. Sci., 10, (2), 117–125.

Vincek D., Gorjanc G., Luković Z., Malovrh Š., Poljak F., Kovač M. (2003): Estimated of genetic parameters for time on test and backfat thickness for gilts from field test. Agriculturae Conspectus Scientificus, 68, (2), 109-113.

Vuković V. (2003): Estimation of genetic parameters for most important reproductive and productive traits of pigs. Doctoral Dissertation. University "St. Cyril and Methodius", Faculty of Agriculture Skopje, 1-115 pp.

Vukovic V., Andonov S., Kovac M., Kocevski D., Malovrh S. (2007): Estimation of genetic parameters for number of teats and traits from performance test of gilts. In: 3rd Joint Meeting of the Network of Universities and Research Institutions of Animal Science of the South Eastern European Countries, Greece, Thessaloniki, February 2007.

Wolf J., Wolfová M. (2012): Impact of genetic groups and herd-year-season fixed/random on genetic parameter estimates from large data sets in pigs. Research in pig breeding, 6, (2), 88-96.

Wongsakajornkit N., Imboonta N. (2015): Genetic correlations among average daily gain, backfat thickness and sow longevity in Landrace and Yorkshire sows. Thai Journal of Veterinary Mededicine, 45, (2), 221-227.

ASSESSMENT OF GENETIC DIVERSITY OF HUNGARIAN OAK (*Quercus frainetto* Ten) AT THE LEVEL OF THE SEED STAND RS-2-2-qfr-00-806

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ABSTRACT

Familiarity with the level of genetic diversity is of particular importance for the process of plant breeding and conservation of available gene pool. Knowledge on the level of the genetic diversity of an individual seed stand is of a great importance when choosing the source of reproductive material. The aim of this research was to determine the structure and genetic diversity of Hungarian oak (*Quercus frainetto* Ten) in the seed stand RS-2-2-qfr-00-806 using SSR (Simple Sequence Repeat) markers. DNA was extracted from the buds of 20 test trees of the Hungarian oak which were sampled during the hibernation. The test trees were evenly distributed throughout seed stand and the distance between them was more than 50 m.

Seven SSR markers were analyzed and all were polymorphic. Total number of alleles revealed for all analyzed individuals were 23, ranging from two (QpZAG112, QpZAG36 and QpZAG15) to five (QpZAG9). Genetic similarities were calculated on binary data using Dice's coefficient by NTSYSpc2.1 program package. High genetic variation was observed among analyzed genotypes, as genetic similarity covered a larger range of values (0.23-0.93).

The obtained level of genetic variability of the seed stand RS-2-2-qfr-00-806 represents a good starting point for future experiments on Hungarian oak breeding. This is basic research for the improvement of mass production of quality reproductive material of Hungarian oak in Serbia.

***Keywords*:** Hungarian oak, SSR markers, population structure, seed stand

INTRODUCTION

The forests as the most complex ecosystems on Earth, are characterized by the high level of diversity in terms of the genetic resources, species abundance and variety of habitats (GEBUREK and KONRAD, 2008). However, the biodiversity is endangered due

to the constant pressure on forests over the last few centuries and the disappearance of natural forests (CARABEO *et al.*, 2016). The long-term survival of species is closely linked to their genetic diversity (GAPARE, 2014). Influenced by the changed environmental conditions, biotic pathogens and damage, the survival and the evolution of species depend on the level of the genetic diversity (REED and FRANKHAM, 2003). The research on the genetic diversity which identifies populations characterized by high genetic variability can help in reducing the risk of losing biological diversity (SOUTO *et al*., 2015).

Quercus frainetto Ten is the oak species that grows in thermophile deciduous forests in south-eastern Europe. The largest distribution is in the Balkan Peninsula, but it also occurs in north-western Turkey and in southern Italy. It grows in a wide vegetation belt rich in woody species. It grows mainly in habitats with sub-continental climate, frequent summer droughts, highest rainfall in spring, wide temperature amplitude, and low winter temperatures. (HORVAT *et al*., 1974). In Serbia, the Hungarian oak grows in the community with the Turkey oak (*Quercetum frainetto-cerridis* Rudski 1949) and represents the zonal forest with the widest distribution that grows at altitude of 600 m. It is in contact with inhabited areas and agricultural land, so its distribution area constantly decreases. In the last century, the areas around the Hungarian oak forests have been significantly reduced due to the anthropogenic influence, and this has had a very negative impact on its genetic diversity.

The Hungarian oak seedlings in Serbia practically have not been produced due to the lack of quality seed and recognized seed sources. Therefore, the afforestation in the oak forest belt, especially in the habitats of the community *Qurcetum frainetto-cerris* Rud. that spatially occupies extensive areas in Serbia, is carried out by alternative species.

The success of afforestation of barren soil and the amelioration of degraded forests depends primarily on the providing of quality seed and planting material. The seed material used for the production has to possess the genetic characteristics that will give the seedlings of desirable morphological characteristics and vitality from which will be established, with adequate tending and protection measures, new forests of a good quality. The seed material of the Hungarian oak from seed stands is the basis for establishing perspective production and ameliorated forests of this species on larger areas. The natural stands of Hungarian oak are mainly of vegetative origin. In order to be a reliable source of planting material, it is necessary to determine their genetic diversity.

The familiarity with the level of genetic diversity is of particular importance for the process of plant breeding and conservation of available gene pool. The knowledge on the genetic diversity' level of an individual seed stand is of a great significance when choosing the source of reproductive material.

The aim of this research was to determine the genetic diversity of the Hungarian oak seed stand RS-2-2-qfr-00-806 using SSR (Simple Sequence Repeat) markers in order to exploit this seed stand in the future as a reliable source of the reproductive material.

MATERIAL AND METHODS

The researches were conducted in the seed stand RS-2-2-qfr-00-806 located in the FMU "Stol" 57/b (X= 4875002; Y=7589431) at the altitude of 240-300 m. The buds necessary for the DNA isolation were sampled during hibernation from the 20 Hungarian oak test trees that are evenly distributed throughout seed stand and the distance between them is more than 50 m. After sampling, the buds were put in plastic bags and transported in the refrigerator to the laboratory.

A total genomic DNA was extracted from the buds of 20 test trees of the Hungarian oak which were sampled during the hibernation using modified CTAB (cetyl trimethylammonium bromide) procedure according to DOYLE and DOYLE (1987), and it was quantified on Eppendorf BioSpectrometer® kinetic. Simple sequence repeat (SSR) characterization was done with seven polymorphic markers previously developed for *Quercus petraea* and *Quercus robur* (Table 1).

Table 1. List of seven informative primers, with their sequences, annealing temperatures, number of alleles and allele range

PROBE	PRIMER SEQUENCES	Annealing temp.	Number of alleles	Allele range (bp)
QpZAG110	F:GGAGGCTTCCTTCAACCTACT R:GATCTCTTGTGTGCTGTATTT	50°C	5	205-260
QpZAG15	F:CGATTTGATAATGACACTATGG R:CATCGACTCATTGTTAAGCAC	52°C	2	110-130
QpZAG36	F:GATCAAAATTTGGAATATTAAGAGAG R:ACTGTGGTGGTGAGTCTAACATGTAG	53°C	2	200-210
QpZAG9	F:GCAATTACAGGCTAGGCTGG R:GTCTGGACCTAGCCCTCATG	55°C	4	190-250
QrZAG108	F:CTAGCCACAATTCAGGAACAG R:CCTCTTTTGTGAATGACCAAG	52°C	4	220-255
QpZAG119	F:GATCAGTGATAGTGCCTCTC R:GATCAACAAGCCCAAGGCAC	53°C	4	65-80
QrZAG112	F:TTCTTGCTTTGGTGCGCG R:GTGGTCAGAGACTCGGTAAGTATTC	51°C	2	95-105

Polymerase chain reaction (PCR) was carried out in 25 µL reaction volume containing: 25 ng of DNA sample, 1 x Buffer (Fermentas), 0.8 mM dNTP, 0.5 µM of each primer pair and 1U Taq polymerase (Fermentas). The PCRs were performed on thermocycler Biometra TProfessional Standard 96 using the following program: an indicial

denaturation at 94ºC/15min. by 32 cycles each of denaturation at 94ºC/45s, annealing at different temperatures (Table 1) for 1 min and extension at 72ºC /45s. Final elongation was at 72°C for 10 min. The amplified PCR fragments were separated on 8% polyacrylamide gel using vertical electrophoresis system (Mini Protean Tetra-Cell BioRad) for 50 min, with 20 bp ladder (Fermentas) as a marker. After staining with 0.5 μg/μL ethidium bromide, gels were photographed under UV light on BioDocAnalyse Biometra. Data were assembled into a binary matrix after SSR profiles were scored as presence or absence (1/0) of alleles in each sample. Genetic similarities (GS) between individuals of *Quercus frainetto* RS-2-2-qfr-00-806 seed stand were calculated in accordance to DICE (1945) in statistical NTSYSpc2 program package (ROHLF, 2000).

RESULTS AND DISCUSSION

Molecular characterization of individual trees of *Quercus frainetto* RS-2-2-qfr-00-806 seed stand was done with seven polymorphic SSRs, in order to evaluate their genetic variability. Due to poor amplification, one individual was excluded from further analysis. Total number of alleles revealed for 19 analysed individuals were 23, ranging between two (QpZAG112, QpZAG36 and QpZAG15) to five (QpZAG110), with average value of 3.28 per locus. The number of alleles and size range were presented in Table 1. LOPEZ-ALJORNA *et al*. (2007) study showed higher number of alleles (9.33) with nine stands of *Quercus suber* from Spain. They observed 20 alleles with QpZAG110, which is similar (19) to work of HERNERO *et al*. (2001) with cork oak from Spain. This could be explained with more stands from different areas of distribution in those studies, contrary to our work with one seed stand and smaller number of individuals.

Based on presence or absence of alleles among analysed individuals, the coefficient of similarity was calculated by Dice (Table 2). The high genetic variability was observed among analysed genotypes, as genetic similarity covered a larger range of values (0.23-0.93). The greatest similarity was found between neighbouring trees, while with increasing distance between the trees the coefficient of similarity decreased. This result could be expected and can be explained by participation of vegetative origin trees in the stand establishment. The average genetic similarity (0.53) shows relatively high level of diversity among the analysed individuals, which is common in Quercus species. Some earlier studies explained high level of intra-population variability due to mating system, low distance among stands and the small size of stands (DOW and ASHLEY, 1998; FINKELDEY, 2001; LOPEZ-ALJORNA *et al*., 2007).

The largest part (32.75%) of obtained GS values was between 0.41 and 0.6, while the lowest GS (0.21-0.4) were represented by 29.24%. On the other hand, the smallest percentage (11%) included GS above 0.76 (Figure 1). The relatively low share of trees with high genetic similarity confirms the good selection and registration of the studied

seed stand and ensures the production of high quality reproductive material. The SSR markers used in this research, although developed for *Q.petrea* and *Q. robur*, proved to be useful in assessing genetic differentiation among individuals of *Quercus frainetto* seed stand.

Table 2. Genetic similarity coefficients by Dice obtained using SSR markers

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1																		
2	0.63	1																	
3	0.37	0.62	1																
4	0.35	0.58	0.94	1															
5	0.5	0.87	0.62	0.58	1														
6	0.5	0.75	0.75	0.7	0.75	1													
7	0.5	0.62	0.5	0.47	0.62	0.5	1												
8	0.42	0.63	0.73	0.7	0.73	0.73	0.63	1											
9	0.62	0.37	0.25	0.23	0.37	0.5	0.25	0.42	1										
10	0.44	0.33	0.22	0.21	0.33	0.44	0.22	0.38	0.77	1									
11	0.5	0.5	0.37	0.35	0.5	0.5	0.37	0.52	0.62	0.66	1								
12	0.5	0.37	0.37	0.35	0.37	0.5	0.37	0.63	0.62	0.66	0.87	1							
13	0.5	0.37	0.25	0.23	0.37	0.37	0.37	0.52	0.62	0.77	0.75	0.87	1						
14	0.35	0.35	0.47	0.44	0.35	0.35	0.35	0.5	0.47	0.52	0.82	0.82	0.7	1					
15	0.53	0.4	0.26	0.25	0.4	0.4	0.26	0.44	0.66	0.82	0.8	0.8	0.93	0.62	1				
16	0.62	0.5	0.37	0.35	0.5	0.62	0.25	0.42	0.87	0.66	0.5	0.5	0.5	0.35	0.53	1			
17	0.44	0.44	0.33	0.31	0.44	0.55	0.22	0.38	0.66	0.9	0.55	0.55	0.66	0.42	0.7	0.77	1		
18	0.5	0.62	0.5	0.47	0.62	0.62	0.37	0.52	0.5	0.55	0.87	0.75	0.62	0.7	0.66	0.62	0.66	1	
19	0.5	0.5	0.37	0.35	0.5	0.5	0.37	0.52	0.5	0.66	0.62	0.75	0.87	0.58	0.8	0.62	0.77	0.75	1

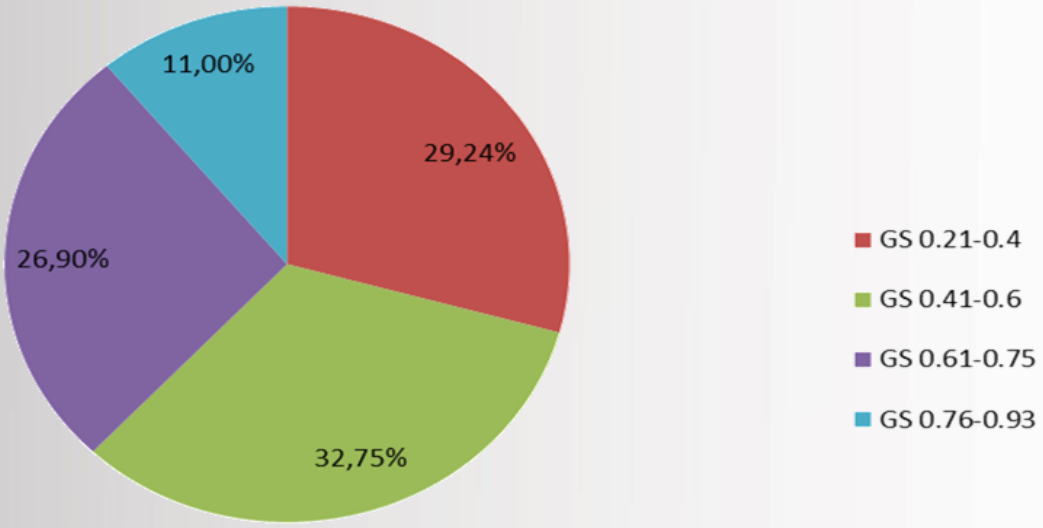

Figure 1. Dispersion of pairwise Dice's genetic similarity values of 19 individuals of *Quercus frainetto* RS-2-2-qfr-00-806 seed stand obtained from SSR data.

CONCLUSION

Basic insight into the level of the genetic diversity of the RS-2-2-qfr-00-806 Hungarian oak seed stand was determined using selected SSR markers. The obtained results show a satisfactory level of the genetic diversity at the level of the studied seed stand. Most of the studied test trees show the coefficient of similarity below 0.75, which can be considered quite sufficient for seed production and utilization. It is necessary to carry out the genetic amelioration in the seed stand where the closest competitors should be removed in order to reduce the number of close relatives. For the confirmation of the obtained results and acceptance of more detailed measures and recommendations in the collection and utilization of seed, the research should also be performed on the progeny produced from the collected seed.

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REFERENCES

Carabeo M., Simeone M. C., Cherubini M., Mattia C., Chiocchini F., Bertini L., Caruso C., La Mantia T., Villani F., Mattionia C. (2016). Estimating the genetic diversity and structure of *Quercus trojana* Webb populations in Italy by SSRs: implications for management and conservation. Can. J. Forest Res. 47: 331-339.

Dice, L.R. (1945): Measures of the amount of ecologic association between species. Ecology, 26: 297-302.

Dow B. D., Ashley M. V. (1998). Levels of Gene Flow in Bur Oak Revealed by Paternity Analysis Using Microsatellites. J. Hered. 89: 62–70.

Doyle J. J., Doyle J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, 19: 11-15.

Finkeldey R. (2001). Genetic variation of oaks (*Quercus* spp.) in Switzerland. 2. Genetic structures in "pure" and "mixed" forests of pedunculate oak (*Q. robur* L.) and sessile oak (*Q. petraea* (MATT.) Liebl.). Silvae Genet. 50 (1): 22-30.

Geburek T., Konrad H. (2008). Why the conservation of forest genetic resources has not worked. Conserv. Biol. 22 (2): 267–274.

Gapare W.J. (2014). Merging applied gene conservation activities with advanced generation breeding initiatives: a case of study of *Pinus radiate* D. Don. New For. 45: 311–331.

Hornero J., Gallego F. J., Martínez I., Toribio M. (2001). Testing the Conservation of *Quercus* spp. Microsatellites in the Cork Oak, *Q. suber* L. Silvae Genet. 50, 3–4.

Horvat L., Glavac V., Ellemberg H. (1974). Vegetation Suedosteuropas. Geobot. Selecta 4. G. Fischer, Stuttgart.

López-Aljorna A., Ángeles Bueno M., Aguinagalde I., Martín J. P. (2007). Fingerprintingand genetic variability in cork oak (*Quercus suber* L.) elite trees using ISSR and SSR markers. Ann. For. Sci. 64 (7): 773-779.

Reed D. H., Frankham R. (2003). Correlation between fitness and genetic diversity. Conserv. Biol. 17: 230–237.

Rohlf F.J. (2000). NTSYS-pc. Numerical taxonomy and multivariate analysis system. Version 2.0 Exeter Software, Setaket, N.Y.

Souto C., Mathiasen P., Acosta M. (2015). Identifying genetic hotspots by mapping molecular diversity of widespread trees: when commonness matters. J. Hered. 106: 537–545.

UTILIZATION AND TRANSFER OF FOREST GENETIC RESOURCES BY INTRODUCTION OF ALIEN SPECIES

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ABSTRACT

Emerging needs for wood, non-wood forest products, different ecosystem services and research programs in the last two centuries have affected the transfer of forest genetic resources within and outside of their natural distribution area. The transfer of species to the new habitats outside their range of distribution involves the well-known risks - reduced growth and (or) dieback as a result of the low adaptive potential of the introduced species to the new environmental conditions. The methods of close and distant - intraspecific and interspecific hybridization have been applied together with the establishment and analysis of the provenance tests throughout the 20th century in Serbia in order to provide a reliable assessment of the adaptive, productive and reproductive potential of the introduced species. The extensive establishment of the plantations for the production of timber for mechanical and chemical wood processing has been one of the main reasons for the introduction of alien species, especially conifers of *Pinus, Picea, Pseudotsuga* genera and the species of *Populus* and *Salix* genera. This paper deals with the attitude of the human population towards the introduction of alien species, their effects on native habitats and indirect influence on the progress of woody plant improvement.

Keywords: Alien species, forest genetic resources

INTRODUCTION

Genetic resources of forest trees have been used and transferred by humans for millennia. For instance, the ancient Greeks and Romans played a significant role in spreading *Castanea sativa* and its cultivation from the Eastern Mediterranean region (including Anatolia and the Caucasus) to other parts of Europe (CONEDERA *et al*., 2004). Over the last 200 years, genetic resources of forest trees have been increasingly transferred, within and outside of species' native distribution ranges, for forestry and for research and development. Transferred germplasm has been used to grow trees for numerous purposes, ranging from the production of wood and non-wood products to the provision of ecosystem services such as the restoration of forests for biodiversity

conservation. International provenance trials have been essential for selecting seed sources for reforestation and for improving tree germplasm through breeding. Many tree breeding programmers were initiated in the 1950s, but as one round of testing and selection typically takes decades, the most advanced of them are only in their third cycle. Recent advances in forest genomics have increased the understanding of the genetic basis of different traits, but it is unlikely that molecular marker-assisted approaches will quickly replace traditional tree breeding methods. Transfers of tree germplasm involve some risks of spreading pests and diseases, introducing invasive tree species and polluting the genetic make-up of already present tree populations. Many of these risks have been underestimated in the past, but they are now better understood and managed. Relatively few tree species used for forestry have become invasive, and the risk of spreading pests and diseases while transferring seed is considerably lower than when moving live plants. This paper attempts to unravel the relationships between humans and woody plants by looking at the importance of introduction of woody plant species for different human activities and especially for forest trees improvement (ISAJEV *et al*., 1986, 2017).

Transfer of forest genetic resources: an historical overview

The history of woody plant introduction is closely linked with that of transportation and European exploration of the planet (16^{th}–19^{th} centuries), (CROSBY, 1986). The transport of a species from one biogeographical region to another was carried out with a particular purpose. Once introduced to a new region, many of these species have been spread un-intentionally by humans within the new biotic regions. Each colonial power established major botanical gardens and experimental stations in various parts of the world, first in the home country and on tropical islands, later in coastal areas and finally in more inland locations.

Ornamentals have been widely introduced in every part of the world. In the past, botanic gardens and individuals were responsible for these introductions. Botanic gardens in all parts of the world have been responsible for the introduction of a large number of species and in every case, species have started to spread into the surrounding vegetation. Erosion control has been a common reason for the introduction of plant species in many parts of the world. Species providing a rapid and thorough cover such as *Lonicera japonica* and *Pueraria lobata* have been favored but these have become major pests (WILLIAMS, 1994). *Elaeagnus angustifolia* was commonly planted as a windbreak and *Lonicera japonica* was planted by game managers for wild deer (BLAISDELL, 1967). In countries such as Britain shrub species such as *Symphoricarpos albus*, were introduced to provide ground cover for game birds (GILBERT, 1995).

The location of introduction was identified, in full or in part, for 388 of the 458 forestry tree species known to occur outside their native range (85 %). Figure 1 shows the number of forestry species that were recorded as introduced, intentionally or by accident, into each of seven geographic regions (Europe, Africa, Australasia, North America, South America, Pacific and Asia). Introductions of forestry species were recorded for all regions.

By 1850, deforestation had reduced average forest cover in Europe to an estimated 20% of land (KAPLAN *et al*., 2009). Already in the late 18th century, several European countries had started largescale reforestation efforts to stop this forest decline and the continent's forest cover subsequently started to increase during the 19th and 20th centuries (MATHER, 2001). By the 20th century, the purpose of introductions shifted from food plants to timber and other species yielding non-agricultural products. Finally, during the latter part of the 20th century the importance of ornamental species increased dramatically, especially to the more developed and wealthier regions.

The transition from deforestation to reforestation created a strong demand for forest tree seed. In many countries, however, the remaining forests could not meet the high demand and seed had to be sourced from other nations. As a result, large quantities of *L. decidua*, *P. abies*, *P. sylvestris* and *Quercus spp*. seed were transferred across Western and Central Europe throughout the 19th century and into the early 20th century (TULSTRUP, 1959). The use of tree species introduced into Europe has also played an important role in these historical reforestation efforts (KJAER *et al*., 2014).

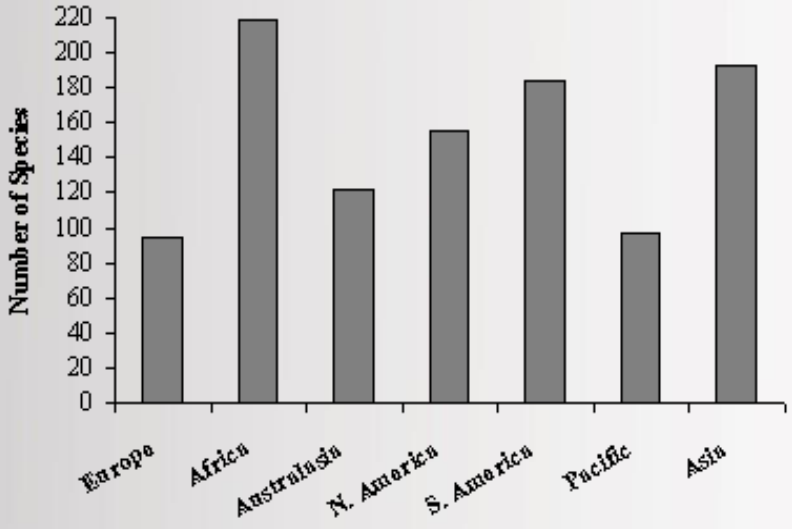

Figure 1. Number of forestry species encountered that having been introduced into each of seven geographic regions, HAYSOM and MURPHY, 2003.

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The environmental risks associated with genetic pollution were largely ignored in the past and it is important not to overstate them now. Strong barriers to hybridization exist between some related species, such as differences in flowering time or the poor fitness of hybrids, which reduce the risks. One approach to reduce the potentially negative impacts of cultivated-wild tree hybridization is to deliberately isolate cultivated material or to plant exotic rather than indigenous trees around natural forests and woodlands (POTTS *et al*., 2001). More research is required on the magnitude of outbreeding depression in tree species, as it remains a relatively understudied phenomenon, with evidence limited mostly to interspecific hybrid segregants (ELLSTRAND, 2003; EDMANDS, 2007). The topic is discussed further in other papers of this special issue (WICKNESWARI *et al*., 2014; THOMAS *et al*., 2014).

Utilization of forest genetic resources research and development

With a few exceptions, forest genetic resources have been utilized extensively in systematic research and development only for about 100 years. The oldest form of research and development is the testing of tree species and their provenances for different uses and under different environmental conditions. The main purpose of provenance research has been, and still is, the identification of well-growing and sufficiently adapted tree populations to serve as seed sources for reforestation (KONIG, 2005). Such research has shown that most tree species have a high degree of phenotypic plasticity (i.e., large variation in phenotype under different environmental conditions (e.g., REHFELDT *et al*., 2002) and that this varies between provenances (e.g., AITKEN *et al*., 2008). Since the 1990s, provenance trials have also demonstrated their value for studying the impacts of climate change on tree growth (e.g., MATYAS, 1994, 1996). Many old provenance trials still exist and continue to provide valuable information for research and development. Due to the long timeframe (often in decades) to reach recommendations, however, it has been challenging for many countries and research organizations to maintain trials and to continue measuring them.

Experience with some conifer exotics in Europe

Tree species, particularly conifers, have been introduced from all over the northern hemisphere into Europe, and this is an unsurpassed region in which to obtain information on the behavior of exotics. It is notable that, after more two centuries of

experience, the majority of foresters in Western Europe are inclined to be pessimistic regarding exotics, because they have not yet found a completely successful introduced tree, even though certain species showed great initial promise. The native European trees grow slowly, so there has been a search for faster growing species. Eastern white pine (*Pinus strobus* L.) grows more rapidly than the native European conifers with which it has been associated. In northern Germany near Eberswalde, the writer has seen 45-year-old Douglas fir (*Pseudotsuga taxifolia* (Lam.) Br.) of the same size as 100-year-old Scots pine. Near Tharandt, 55-year-old Douglas firs were from 14 to 20 inches (36 to 51 cm.) in diameter at breast height, exactly twice the size of Norway spruce (*Picea abies* Karst.) of the same age mixed with them. In southern Germany, near Munich, 45-year-old planted Douglas fir was the same height as 77-year-old naturally reproduced silver fir (*Abies alba* Mill.).

Overview of domestication and conservation approaches of poplar (Populus L.) and willows (Salix L.)

The natural range of *Populus* and *Salix* spans impressive ecological amplitude, primarily across the North American, European and Asian land masses – from the subtropics to the boreal forests and arctic tundra, riparian to montane ecosystems and the man-made environment of modern agriculture. As a consequence, poplar and willow geneticists – those responsible for conserving and domesticating germplasm of *Populus* and *Salix* – have an especially broad mandate: to study the genetic diversity of natural populations and be familiar with all the modern tools for genetic improvement in order to serve specific social needs (KUZOVKINA and QUIGLEY, 2005; KUZOVKINA *et al*., 2008; KUZOVKINA and VOLK, 2009; STANTON *et al*., 2010).

Populus domestication has a history of nearly 100 years, beginning with Henry's work-1914, at the Royal Botanic Gardens, Kew, in the UK, and the work of STOUT and SCHREINER, 1933 and STOUT *et al*. 1927 at the New York Botanical Garden in the USA. Other early domestication efforts include those of WETTSTEIN-WESTERSHEIM 1933 in Germany, AL'BENSKII and DELITSINA (1934) in Russia, HEIMBURGER (1936) in Canada, and HOUTZAGERS (1952) in the Netherlands. *Salix* domestication traces to the hybridization studies of Heribert-Nilsson in Sweden, (HERIBERT-NILSSON, 1918), along with Nilsson and Hakansson's cytological work in the 1930s, (NILSSON, 1931; HAKANSSON, 1933, 1938). In the UK, H.P. Hutchinson began work in willow conservation and breeding in the 1920s at the Long Ashton Research Station that was continued by K.G. Stott for the following 30 years (NEWSHOLME, 1992; STOTT, 1992). Most of this work involves 12 species in the genus *Populus* that are noteworthy for their commercial and ecological values. They are the North American species *P. balsamifera*, *P. deltoides*, *P. trichocarpa* and *P. tremuloides* and the Eurasian species *P. alba*, *P. cathayana*, *P. ciliata*, *P. euphratica*, *P. maximowiczii*, *P. nigra*, *P. simonii* and *P. tremula* (STANTON *et al*., 2014).

Within the genus *Salix*, 10 species − *S. caprea*, *S. dasyclados*, *S. eriocephala*, *S. koriyanagi*, *S. miyabeana*, *S. purpurea*, *S. udensis*, *S. schwerinii*, *S. triandra* and *S. viminalis* − are being utilized in developing the world's renewable energy industry, while three others − *S. alba*, *S. babylonica* (synonym *S. matsudana*) and *S. nigra* − are favored for timber products (STANTON *et al*., 2014).

Experience with black locust (Robinia pseudoacacia L.)

The North American black locust has been widely introduced throughout Europe as a source of high quality timber and for erosion control. Some now regard it as a permanent member of the flora (GAMS, 1967). The main uses of *R. pseudoacacia* have been somewhat variable in different parts of Europe and have changed over time. In parts of France and Switzerland the young coppice wood was extensively used in vineyards to support the vines (MONNIER, 1992), but in recent decades it has been replaced by metal posts and wire, and now the species is hardly used. Although the tree produces a highly durable timber, it is disliked by German foresters because the wrong strain, a shrubby variety, was introduced to that country. In Hungary the tree has remained a key timber and is the main source of honey (KERESZTESI, 1977).

Testing and evaluating of introduced exotic species

Introduction of exotic tree species has been the single most important aspect of forest tree improvement for some areas. On the other hand, some regions have such excellent native species or such harsh growing conditions that trees from other lands have proved of little value. There are various intermediate regions in which tree introduction is on of several improvement methods which should be considered.

The procedures and designs used to test exotics are the same as used in the study of individual tree inheritance and racial variation. Exotic testing should be done in two or three stages (WRIGHT, 1993). The first preliminary test should include several scattered plantations on different soil types and with different climates, with few blocks per plantation and small plots. These first tests may well include a few hundred seedlots of several different species or even genera. They may be established over a period of years, with separate plantations for species with different growth rates and growth habits. The second-stage test should concentrate on those races or species that grew best in the first-stage tests. There should be more replication and perhaps larger plots at each test site, and increased attention can be given to individual tree variation. The second-stage trails may often be considered as semi-commercial, designed for the production of wood, as well as data. The third-stage trails can consist commercial plantations designed primarily for wood production.

When working with exotic species for the first time there are lacks the background information of site adaptability, pest problems and silviculturals management that is usually available for a native species. This is the reason for

suggesting preliminary testing of variety of site conditions and moderate-scale second-stage testing.

Identification and monitoring of introduced species should be particularly supported in those areas where currently is a lack of documentation. A number of case studies should be conducted in collaboration with countries that have a high degree of dependence on forestry. Such case studies should cover a range of forestry situations (commercial, developmental and environmental) and include the development and promotion of tools for ecological and economic impact assessments. These could also be incorporated into more general decision support systems that include socio-economic factors, as well as biological risk.

CONCLUSIONS

Advances in research and development work in the forestry sector in different parts of the world have shifted germplasm demand toward species and provenances expected to perform well at specific sites for particular functions, bringing significant productivity benefits.

There is a need for further research and monitoring that will provide information on the management processes in planted systems and take account of the scale (i.e. land area) of plantings and of the area occupied by introduced species.

- Introductions should only be considered if clear and well-defined benefits to man or natural communities can be foreseen and demonstrated.

- Introductions should only be considered if no native species are suitable for the purpose for which the introduction is being made.

- Introductions should not be made into pristine natural or semi-natural habitats, reserves of any kind or their buffer zones and, in most cases, oceanic islands.

- The taxonomic identification of the proposed introduction needs to be confirmed.

Only if these first four conditions are met should further assessment be undertaken. Generally, it may be accepted conclusion, that introduced species can fulfill a gradually increasing role in certain ecological or industrial niches but will probably not replace natives over large areas (WRIGHT, 1993).

The continued need for germplasm transfers for research is well recognized by scientists and research institutes who are pressing their governments to minimize the bureaucracy and costs related to the implementation of the Nagoya Protocol.

REFERENCES

Aitken S.N., Yeaman S., Holliday J.A., Wang T., Curtis-McLane S. (2008). Adaptation, migration or extirpation: climate change outcomes for tree populations. Evol.Appl. 1, 95–111.

Al'benskii A.V., Delitsina, A.V. (1934). Experiments and investigations. All-Union Institute of Forest Cultivation and Forest Melioration, Moscow, Russia. 2nd Issue, pp. 107–109 (*Plant Breeding Abstracts* 5, 62)

Blaisdell J.P. (1967). Introduction of wild-land plants into the United States – methods, legal controls, and ecological implications. IUCN Publication NS 9: 13–26.

Conedera M., Krebs P., Tinner W., Pradella M., Torriani D. (2004). The cultivation of *Castanea sativa* (Mill.) in Europe, from its origin to its diffusion on a continental scale. Veg. Hist. Archaeobot. 13, 161–179.

Crosby A.W. (1986). Ecological Imperialism: The Biological Expansion of Europe, 900–1900. Cambridge University Press, UK.

Edmands S. (2007). Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. Mol. Ecol. 16,463–475.

Ellstrand N.C. (2003). Dangerous Liaisons? When Cultivated Plants Mate With Their Wild Relatives. John Hopkins University Press, Baltimore, Maryland, USA

FAO (1996). International standards for phytosanitary measures: guidelines for pest risk analysis. Publication No. 2. Rome, Italy, Secretariat of the International Plant Protection Convention.

Gams H. (1967). Introduction and spread of 'weed' plants in Europe. IUCN Publication NS 9: 72–75.

Gilbert O.L. (1995). Biological Flora of the British Isles. *Symphoricarpos albus* (L.) S.F. Blake (S. *rivularis* Suksd., *S. racemosus* Michaux). Journal of Ecology 83: 159–166.

Hakansson A. (1933). The conjugation of chromosomes in a *Salix* hybrid. Hereditas 18, 199–214.

Hakansson A. (1938). Cytological studies on bastards of *Salix*. Hereditas 24, 1–32.

Heimburger C. (1936). Report on poplar hybridization 1936. The Forestry Chronicle 12, 285–290.

Heribert-Nilsson N. (1918). Experimentelle studien uber variabilitat, Spaltung, Artibildung, und evoluation in der gattung *Salix*. Lunds Universitats Arsskrift N. F.Avd 2.

Houtzagers G. (1952). Forest genetics and poplar breeding in the Netherlands. II. Poplar breeding in the Netherlands. *Euphytica* 1, 161–174.

Haysom K.A., Murphy S.T. (2003). The status of invasiveness of forest tree species outside their natural habitat: a global review and discussion paper. Forest Resources Development Service Working Paper FBS/3E, Forest Resources Division,FAO,Rome,Italy.

Isajev V., Tucović, A. (1986). The importance and the application of introduction in Genetics and tree and shrub improvement. Bulletin of the Faculty of Forestry 67, University of Belgrade - Faculty of Forestry, Belgrade, pp 19-28.

Isajev V., Lučić A., Popović V., Stanković-Nedić M. (2017). Značaj introdukcije drvenastih biljaka za oplemenjivanje šumskog drveća. ŠUMARSTVO 3-4. pp, 9-24.

Kaplan J.O., Krumhardt K.M., Zimmermann N. (2009). The prehistoric and preindustrial deforestation in Europe. Q. Sci. Rev. 28, 3016–3034.

Koskela J., Vinceti B., Dvorak W., Bush D, Dawson I. K., Loo J., Kjaer E. D., Navarro C., Padolina C., Bordacs S., Jamnadass R., Lars Graudal L., Ramamonjisoa L. (2014). Utilization and transfer of forest genetic resources: A global review. Forest Ecology and Management 333 pp. 22–34.

Keresztesi B. (1977). *Robinia pseudoacacia*: the basis of commercial honey production in Hungary. Bee World 58: 144–150. Khalanski, M. 1997.

Kjaer E.D., Lobo A., Myking T. (2014). The role of exotic tree species in Nordic forestry. Scand. J. Forest Res. 29, 323–332

Konig A.O. (2005). Provenance research: evaluating the spatial pattern of genetic variation. In: Geburek, T., Turok, J. (Eds.), Conservation and Management of Forest Genetic Resources in Europe. Arbora Publishers, Zvolen, Slovakia, pp. 275–333.

Kuzovkina Y.A., Quigley M.F. (2005). Willows beyond wetlands: uses of *Salix* L. species for environmental projects. Water, Air, and Soil Pollution 162, 183–204.

Kuzovkina Y.A., Volk T.A. (2009). The characterization of willow (*Salix* L.) varieties for use in ecological engineering applications: co-ordination of structure, function and autecology. Ecological Engineering 35, 1178–1189.

Kuzovkina Y.A., Weih, M. Romero M.A., Charles J., Hurst S., McIvor I. (2008). *Salix*: botany and global horticulture. In: Janick, J. (ed.) Horticultural Reviews. John Wiley and Sons, Hoboken, New Jersey, pp. 447–489.

Matyas C. (1994). Modelling climate change effects with provenance test data. Tree Physiol. 14, 797–804.

Matyas C. (1996). Climatic adaptation of trees: rediscovering provenance tests. Euphytica 92, 45–54.

Mather A. (2001). The transition from deforestation to reforestation in Europe. In: Angelsen A., Kaimowitz D. (Eds.), Agricultural Technologies and Tropical Deforestation. CAB International, Wallingford, UK, pp. 35–52.

Monnier Y. (1992). L'empreinte des Amériques dans le paysage: de l'insolite au familier. Cahier d'Outre-Mer 45(179–180): 441–460.

Newsholme C. (1992). Willows: the Genus *Salix.* Timber Press, Portland, Oregon.

Potts B.M., Barbour R.C., Hingston A.B. (2001). The Risk of Genetic Pollution from Farm Forestry Using Eucalypt Species and Hybrids. Rural Industries Research and Development Corporation, Kingston, Australia.

Richardson D.M., Pysek P., Rejmanek M., Barbour M.G., Panetta F.D., West C.J. (2000). Naturalization and invasion of alien plants: concepts and definitions. Diversity and Distributions 6: 93–107.

Rehfeldt G.E., Tchebakova N.M., Parfenova Y.I., Wykoff W.R., Kuzmina N.A., Milyutin L.I. (2002). Intraspecific responses to climate in *Pinus sylvestris*. Glob. Change Biol. 8, 912–929.

Stanton B.J., Neale D.B., Li S. (2010). *Populus* breeding: from the classical to the genomic approach. In: Jansson, S., Bhalerao, R.P. and Groover, A.T. (eds) Genetics and Genomics of *Populus*. Springer, New York, pp. 309–348.

Stott K.G. (1992). Willows in the service of man. Proceedings of the Royal Society of Edinburgh 98B, 169–182.

Stout A.B., Schreiner E.J. (1933). Results of a project in hybridizing poplars. Journal of Heredity 24, 216–229.

Tulstrup N.P. (1959). International trade in forest tree seed. Unasylva 13 (4), 196–201.

Thomas E., Jalonen R., Loo J., Boshier D., Gallo L., Cavers S., Bordacs S., Smith P., Bozzano M. (2014). Genetic considerations in ecosystem restoration using native tree species. For. Ecol. Manage. 333, 66–75.

Wettstein-Westersheim W. (1933). Die kreuzungsmethode und die beschreibung von F1 – bastarden bei *Populus*. [The method of hybridization and the description of F1 hybrids of poplars.] Zeitschrift fur Zuchtung 18, 597–626. (*Plant Breeding Abstracts* 4, 163–164.)

Williams D.F. (ed.). (1994). Exotic Ants: Biology, Impact, and Control of Introduced Species. Westview Press, Boulder, USA.

Wright J.W. (1993). Introduction to Forest Genetics. Academic Press, INC. San Diego, California. pp.359-399 ISBN 0-12-765250-7.

Wickneswari R., Rajora O.P., Finkeldey R., Aravanopoulo F., Bouvet J.-M., Vaillancourt R.E., Kanashiro M., Fady B., Tomita M., Vinson C. (2014). Genetic effects of forest management practices: Global synthesis and perspectives. For. Ecol. Manage. 333, 52–65.

MOLECULAR CHARACTERISATION OF SOYBEAN VARIETIES BY SSR MARKERS

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Soybean (*Glycine max* (L.) Merr.) is one of the most economically important legumes. As the source of plant protein and vegetable oil it is used as food and industrial crop in many regions of the world. The genetic base of soybean cultivars is highly narrow, corresponding to the fact that it is largely a self-pollinated species. Twelve soybean varieties were evaluated with SSR (*Simple Sequence Repeat*) markers selected based on their distribution on the 20 genetic linkage groups. Out of 36 SSR markers, 33 markers were found polymorphic among analyzed genotypes. Total number of alleles was 88, ranging between two and four with an average of 2.67 alleles per marker. The polymorphic information content (PIC) ranged from 0.153 (*Satt229, Satt239 and Satt327*) to 0.775 (*Satt276).* Simple matching similarity coefficient was calculated using NTSYSpc2 program package. The average genetic similarity coefficient for all pairwise was 0.57, with highest value (0.84) between *Galina* and *Lela,* while the lowest value (0.46) was found between *Bosa* and *Nena.* Dendrogram by the UPGMA (*Unweighted Pair Group Method with Arithmetic Mean)* method was constructed on the basis of genetic similarity matrix. Genotypes were distributed in two groups and one branch, mostly in accordance with their pedigree.

***Keywords*:** genetic similarity, SSR, *Glycine max*

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is one of the most economically important legumes. As the source of plant protein and vegetable oil it is used as food and industrial crop in many regions of the world. The genetic base of soybean cultivars is highly narrow, corresponding to the fact that it is largely a self-pollinated species with limited out-crossing. Estimation of the genetic diversity of soybean germplasm is imperative to broaden the genetic base and improve breeding programs (NELSON, 2011). Further progress in soybean breeding requires more intensive utilization of existing genetic resources, and that is application of classical breeding and molecular technologies (VRATARIC and SUDARIC, 2008).

Estimation of genetic diversity can be assessed by the differences in morphological and agronomic traits, pedigree information, geographic origins and molecular markers. In the last two decades genetic diversity methods based on phenotypic traits, morphological and pedigree data were improved with the use of DNA markers, with a variety of different techniques (SPOONER *et al*., 2005). Restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) have been used in soybean's molecular characterisation, including both the advantages and disadventages (POWELL *et al*., 1996; THOMPSON *et al*., 1998; NARVEL *et al*., 2000; UDE *et al*., 2003; LI *et al*., 2010).

Simple sequence repeats are the most frequently and successfully used PCR markers in soybean diversity studies due to their high informativeness, high allelic diversity reproducibility, genetically codominant nature and simple application. SSR makers also important in identifying quantitative trait loci (QTL), which can be associated with certain phenotypic traits of individuals, allowing more efficient selection. SNP marker technology based on DNA sequencing can also be used for evaluating genetic diversity, but they have to be adjusted for divergence studies. (YANG *et al*., 2011).

Soybean cultivars grown in Serbia have mainly originated from closely related genotypes, existing in local germplasm collections which are adapted to similar environmental conditions (PERIĆ *et al*., 2014). The objective of this work was to study genetic diversity and relationships of soybean varieties belonging to different breeding programs using molecular (SSR) approache.

MATERIALS AND METHODS

Twelve soybean varieties (Table 1) were evaluated with SSR (Simple Sequence Repeat) markers and each variety was presented with 20 plants.

Table 1. List of 12 soybean varietes used for SSR characterization

Genotype	Pedigree
AFRODITA	S1346/Hodgson
ZPS 015	NBSG1 population (USA)
LAURA	Kunitz/Novka
VOJVOĐANKA	S1346/Hodgson
LANA	Kunitz/Kador
OLGA	OS101/ZPS208 (=Hobbit/Platte)
LIDIJA	(Sibley/A1937)/Kunitz(Sibley=(Evans/Steele)/Hodgson
NENA	OS101/Elf
SELENA	Afrodita/L9073 (Weber/Kador)
BOSA	Weber/Dawson
LELA	Sava/Zen (Sava =(Balkan/Vojvodjanka)x(Afrodita/Gema))
GALINA	Balkan/Ravnica (Balkan=(Evans/Four)/S1346; Ravnica=Hodgson/S1346)

A total genomic DNA was isolated from young leaves of each variety using *GeneJET™ Plant Genomic DNA Purification Mini Kit*. SSR characterization was done with 36, but only 33 were chosen for statistical analysis (Table 2). Polymerase chain reaction (PCR) was carried out in 20 µL reaction volume containing: 1 x Buffer (*Fermentas*), 0.8 mM dNTP, 0.5 µM of each primer pair, 1U Taq polymerase (*Fermentas*) and 1µL of DNA sample. The PCRs were performed on thermocycler *Biometra TProfessional Standard 96* using the following programme: an initial denaturation at 94ºC/5min., followed by 36 cycles each of denaturation at 94ºC/30s, annealing at temperatures ranging from 49ºC to 63ºC (Table 2) for 45 seconds and extension at 72ºC /1min. Final elongation was at 72°C for 10 min. Afterwards, PCR fragments were separated on 8% polyacrylamide gel in 0.5xTBE buffer using vertical electrophoresis system (*Mini Protean Tetra-Cell BioRad*) for 50 min, with 20 bp ladder (*Fermentas*) as a marker. Gels were photographed under UV light on *BDA live system Biometra* after staining with 0.5 µg/µL ethidium bromide.

Genetic similarities were calculated on binary data (presence or absence of alleles in each sample) using simple matching coefficient (SOKAL and MICHENER, 1958) in statistical NTSYSpc2 program package (ROHLF F.J., 2000). Cluster analysis was performed by UPGMA (*Unweighted Pair Group Method with Arithmetic Mean)* and relationships among varieties were visualized as dendrogram. Poymorphism information content (PIC) for each marker was calculated by the formula: $1-\sum(Pi^2)$, where Pi is the frequency of i-th allele in a locus (LYNCH and WALSH, 1998). PIC value provides information of the discriminating power of locus by taking into account the number of alleles that are expressed and their relative frequencies.

Table 2. List of 33 SSR primers, with their chromosome position, number of alleles, allele range and PIC

Probe	No. of alleles	Chromosome	Allele size (bp)	PIC
Satt 144	2	13	80-120	0.445
Satt 153	4	10	200-260	0.674
Satt 168	2	14	200-260	0.279
Satt 229	2	19	180-250	0.153
Satt 239	2	20	180-200	0.153
Satt 243	3	9	250-300	0.652
Satt 244	3	16	160-220	0.403
Satt 251	2	11	200-220	0.375
Satt 264	3	9	200-280	0.571
Satt 268	4	15	200-280	0.599
Satt 271	2	2	110-140	0.445
Satt 276	4	5	280-320	0.775

Satt 287	3	16	200-250	0.528
Satt 292	3	20	220-280	0.292
Satt 312	2	6	280-300	0.486
Satt 319	2	6	180-200	0.486
Satt 322	3	6	160-220	0.571
Satt 324	2	18	200-260	0.326
Satt 327	2	8	210-280	0.153
Satt 373	4	19	200-260	0.654
Satt 431	3	16	200-250	0.502
Satt 442	2	12	200-280	0.279
Satt 453	3	11	220-280	0.569
Satt 468	3	1	180-220	0.487
Satt 472	3	18	200-300	0.569
Satt 483	3	15	240-280	0.653
Satt 509	3	11	180-220	0.487
Satt 510	2	13	100-140	0.5
Satt 540	3	7	140-180	0.667
Satt 546	2	2	200-250	0.445
Satt 551	2	7	240-260	0.445
Satt 578	2	4	140-200	0.298
Satt 646	3	4	180-220	0.625
Mean	2.67	-	-	0.471

RESULTS AND DISCUSSION

Genetic relationships among soybean varieties could be relevant for future breeding achievement for yield, quality improvement and pest resistance. Integrated genetic evaluation of soybean varieties could facilitate introgression of diverse germplasm into the commercial soybean genetic base (TARA SATYAVATHI *et al*., 2006).

Genetic characterization of 12 soybean varietes was done using 36 SSR markers distributed across different chromosomes (Table 2). Three primers were monomorphic (identical for all analyzed genotypes) and were not included in further data analysis. Total of 88 alleles among the analysed soybean varietes were identified on polyacrylamide gels. The number of alleles varied from two (Satt 144, Satt 168, Satt 229, Satt 239, Satt 251, Satt 271, Satt 312, Satt 319, Satt 324, Satt 327, Satt 442, Satt 442, Satt 510, Satt 546, Satt 551 and Satt 578) to four (Satt 153, Satt 268, Satt 276 and Satt 373) with the average of 2.67 per locus. Similar mean value of alleles per marker (2.97) was obtained in the work of KUMAWAT *et al*. (2015), while a higer value (4.28) was found in the study of TANTASAWAT et al. (2011).

The PIC values ranged from 0.153 (Satt 229, Satt 239 and Satt 327) to 0.775 (Satt 276), with the mean value of 0.471. As the PIC values for 26 markers were > 0.3, it can be concluded that most of the markers were highly informative and could therefore

be considered suitable for molecular characterization of soybean genotypes. Diversity scores reported in other studies were in agreement with this result. In the study of SINGH *et al*. (2010) PIC value was 0.5 and slightly lower (0.48) in the work of KUMAWAT *et al*. (2015). On the other hand, PIC values higher than 0.6 were noted in other works (HUDCOVICOVÁ and KRAIC 2003; LI *et al*., 2010; TANTASAWAT *et al*., 2011). The pairwise genetic similarity (GS) values were relatively high and ranged from 0.46 (between Bosa and Nena) to 0.84 (between Lela and Galina).

The mean value of 0.57 is reflecting a slightly higher degree of genetic diversity among soybean cultivars, compared with previous results (0.65) in work of PERIC *et al*. (2014). The average GS observed in this study was lower compared to those described in similar study with 25 soybean genotypes in Thailand (TANTASAWAT *et al*., 2011). On the other hand, PRIOLI *et al*. (2010) reporeted considerable diversity (GS=0.26) among 168 Brazilian soybean cultivars.

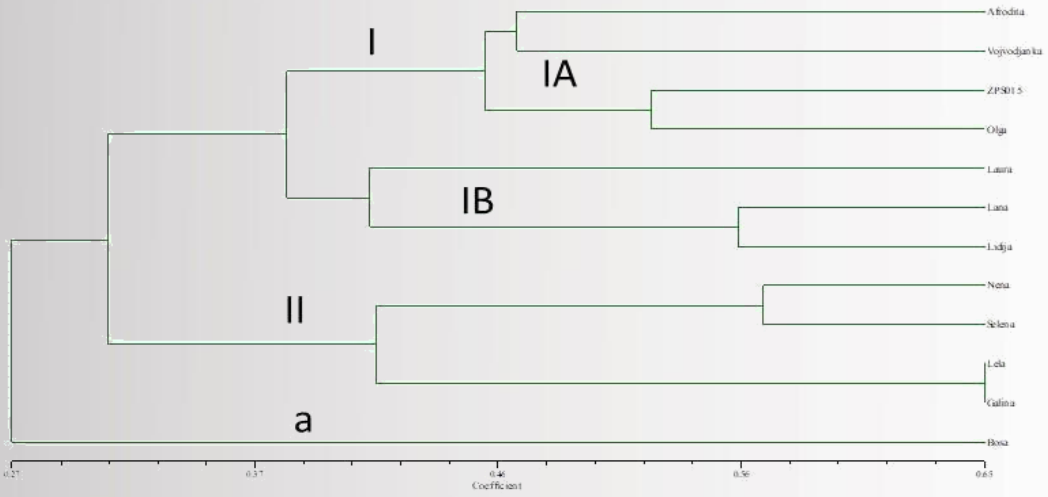

Figure 1. Dendrogram of 12 soybean varieties constructed using UPGMA cluster analysis of simple matching similarity coefficient obtained by SSR data.

The result of cluster analysis based on simple matching genetic similarities was presented in the form of dendrogram (Figure 1). Information about pedigree data of soybean cultivars was crucial for the clarification of the cluster analysis based on SSR data. Two major clusters (I and II) were formed, as well as one attached genotype - branch a. Most of the genotypes (seven) formed cluster I and four formed cluster II. Cluster I was divided into two subclusters. Subcluster IA included genotypes Afrodita and Vojvodjanka, orginating from the same cross, as well as genotypes ZPS015 and Olga, orginating from USA gemplasm. Varietes Laura, Lana and Lidija were grouped together in subcluster IB, originating directly from cultivar Kunitz; therefore, subcluster IB presents a clear example of close positioning of genotypes with the same origin in the

same subcluster. The most heterogeneous was cluster II which included genotypes with different genetic backgrounds, but also some of lines (Selena, Lela and Galina) share a common genetic basis (Hodgson and S1346). Variety Bosa did not group with any of the analyzed genotypes and formed branch *a*. The cluster analysis showed accordance between grouping of genotypes and their pedigree data. Similar results were find in other papers (PERIC *et al*., 2014; BISEN *et al*., 2015; KUJANE *et al*., 2019), comfirming that selection of elite breeding material leading to uniformity.

CONCLUSIONS

The SSR markers used in this work could successfully be applied for genetic diversity researches in soybean cultivars, showing satisfying polymorphism (PIC=0.471). It was found that there was a certain level of agreement between grouping of individual genotypes based on pedigree, but mostly at single pairs and small groups. However, there were also rare cases of individual genotypes founding themselves at greater distances, while according to pedigree data they should share a similar genetic basis.

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REFERENCES

Bisen A., Khare D., Nair P., Tripathi N. (2015) SSR analysis of 38 genotypes of soybean (*Glycine Max* (L.) Merr.) genetic diversity in India. Physiology and Molecular Biology of Plants, 21 (1): 109-115.

Hudcovicová M., Kraic J.(2003) Utilisation of SSRs for Characterisation of the Soybean (*Glycine max* (L.) Merr.) Genetic Resources. Czech Journal od Genetics in Plant Breeding, 39 (4): 120–126.

KujaneK., Sedibe M. M., Mofokeng A. (2019) Genetic diversity analysis of soybean (*Glycine max* (L.) Merr.) genotypes making use of SSR markers. Australian journal of crop science, 13 (07):1113-1119

Kumawat G., Singh G., Gireesh C., Shivakumar M., Arya M., Agarwal D.K., Husain S.M. (2015) Molecular characterization and genetic diversity analysis of soybean (*Glycine max* (L.) Merr.) germplasm accessions in India. Physiology and Molecular Biology of Plants, 21 (1): 101-107.

Li Y.-H., Li W., Zhang C., Yang L., Chang R.-Z., Gaut B.S., Qiu L.-J. (2010): Genetic diversity in domesticated soybean (*Glycine max*) and its wild progenitor (*Glycine soja*) for simple sequence repeat and single-nucleotide polymorphism loci. New Phytologist, 188(1): 242-253.

Lynch M., Walsh J.B. (1998) Genetics and Analysis of Quantitative Traits. Sinauer Assocs., Inc., Sunderland, MA.

Narvel J.M., Fehr W.R., Chu W.C., Grant D., Shoemaker R.C. (2000) Simple sequence repeat diversity among soybean plant introductions and elite genotypes. Crop Science, 40(5): 1452-1458.

Nelson R.L. (2011): Managing self-pollinated germplasm collections to maximize utilisation. Plant Genetic Resources of Canada: Characterization and Utilization, 9(1): 123-132.

Perić V., Nikolić A., Babić V., Sudarić A., Srebrić M., Đorđević V., Mladenović Drinić S. (2014) Genetic relatedness of soybean genotypes based on agromorphological traits and RAPD markers. Genetika, 46(3): 839- 854.

Powell W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey S., Rafalski A. (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for gemplasm analysis. Molecular Breeding, 2(3): 225-238.

Priolli R.H.G., Pinheiro J.B., Zucchi M.I., Bajay M.M.& Vello N.A. (2010) Genetic diversity among Brazilian soybean cultivars based on SSR loci and pedigree data. Brazillian Archives of Biology and Technology, 53(3): 519-531.

Rohlf F.J. (2000) NTSYS-Pc: Numerical Taxonomy and Multivariate Analysis System. Version 2.1., Exeter Publishing Led., New York.

Singh R.K., Bhatia V.S., Bhat K.V., Mohapatra T., Singh N.K., Bansal K.C., Koundal K.R. (2010) SSR and AFLP based genetic diversity of soybean germplasm differing in photoperiod sensitivity. Genetics andMolecular Biology, 33(2):319–324.

Sokal, R.R. Michener C.D. (1958): A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. 38: 1409-1438.

Spooner D., van Treuren R., De Vicente M.C. (2005) Molecular markers for genebank management, IPGRI Technical Bulletin No. 10, International Plant Genetic Resources Institute, Rome, Italy.

Tantasawat P., Trongchuen J., Prajongjai T., Jenweerawat S., Chaowiset W. (2011) SSR analysis of soybean (*Glycine max* (L.) Merr.) genetic relationship and variety identification in Thailand. Australian Journal of Crop Science, 5 (3): 283-290.

Tara Satyavathi C, Bhat K.V., Bharadwaj C., Tiwari S.P., Chaudhury V.K. (2006) AFLP analysis of genetic diversity in Indian soybean [*Glycine max* (L.) Merr.] varieties. Genetic Resources and Crop Evolution, 53(5): 1069-1079.

Thompson J.A., Nelson R.L., Vodkin L.O. (1998) Identification of diverse soybean germplasm using RAPD markers. Crop Science, 38(5): 1348-1355.

Ude G.N., Kenworthy W.J., Costa J.M., Cregan P.B., Alvernaz J. (2003): Genetic diversity of soybean cultivars from China, Japan, North America, and North American ancestral lines determined by amplified fragment length polymorphism. Crop Science, 43: 1858-1867.

Vratarić, M., Sudarić A. (2008) Soja (Glycine max (L) Merr.). Poljoprivredni institut, Osijek, Hrvatska.

Yang X., Xu Y., Shah T., Li H., Han Z., Li J., Yan J. (2011) Comparison of SSRs and SNPs in assessment of genetic relatedness in maize. Genetica, 139(8):1045–1054.

DIFFERENCES BETWEEN DURUM AND COMMON WHEAT GENOTYPES ACCORDING TO THE INSTRUMENTAL AND SENSORY PROPERTIES OF BREAD

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ABSTRACT

In order to determine effects of a genotype on instrumental and sensory properties of bread, two common and two durum wheat genotypes cultivated in two growing seasons (2010 and 2011) were used. Bread loaf volumes and specific volumes were determined by VolScan profiler and results showed that the loaf volume ranged from 186.90 ml to 327.50 ml and from 160.10 ml to 228.10 ml in common and durum wheat bread samples, respectively. The comparison of the bread volume of common and durum wheat genotypes revealed that the bread volume of durum wheat genotypes with the best performance was significantly lower than that of common wheat genotypes. Bread samples made from common and durum wheat genotypes with the smallest specific volume were distinguished by the highest bread crumb firmness and chewiness. The firmness, chewiness, cohesiveness, resilience and springiness of bread crumbs were instrumentally recorded on the TA.XTplus texture analyser. The smallest crumb firmness was registered in the bread made from the genotype ZP Zemunska rosa (395.6 g) grown in the rainy season, while the bread made from the same genotype cultivated during the dry season had 5.4 fold firmer bread crumb. The smallest chewiness was detected in bread samples made from the common wheat genotype ZP 87/I (213.3g) and the durum wheat genotype DSP/01 (211.3g). Results of the sensory evaluation revealed that the sensory properties shape, crumb pore uniformity and structure, varied greatly among the investigated bread samples made from common and durum wheat genotypes. It can be concluded that investigated common and durum genotypes have quite different physical and sensory characteristics which could allow various possibilities of their use.

Keywords: Common and durum wheat, instrumental properties, sensory properties

INTRODUCTION

Wheat is one of the most important cereal crops worldwide in terms of production and utilization and traditionally has been selected for its technological functionality resulting

in the selection of common (*Triticum aestivum* L.) and durum (*Triticum durum* Desf.) wheat varieties. Wheat grains can be ground into flour or semolina, which forms the basic ingredients to prepare bread, pasta, chapati, noodles, cakes, pizzas, and doughnuts. Basically, in one form or another, wherever we are in the world, we consume wheat at every meal.

The differences between common wheat (a.k.a. bread wheat) and durum wheat can be attributed largely to their gluten protein properties. Common wheat gluten proteins upon hydration and mixing, form a strong, cohesive, viscoelastic network that allows the wheat flour dough to retain yeast fermentation gases and to produce a light, aerated baked product (VERAVERBEKE and DELCOUR, 2002). On the other hand, durum wheat normally has weaker and less extensible gluten characteristics than common wheat (EDWARDS *et al*., 2001). Durum wheat has found traditional use in flat breads and specialty breads, particularly in the Mediterranean countries, but in the last 20 year it is also experiencing an increasing application in the Mediterranean region for breads of all types (PALUMBO *et al*., 2000).

The objectives of this work were to examine the differences in instrumental and sensory properties of breads made from two common and two durum wheat genotypes.

MATERIAL AND METHODS

Two common wheat (*Triticum aestivum* L.) and two durum wheat (*Triticum durum* Desf.) genotypes of different origin, pedigrees and a growth type were used in the study. The genotypes were chosen on the basis of differences in agronomic traits such as yield and its components. Grain samples of common and durum wheat were collected from plants grown in a variety trial at the Maize Research Institute, Zemun Polje (MRIZP) in 2010 and 2011 growing season. Standard cropping practices were applied to provide adequate nutrition and to keep the disease- and weed-free plots. Common and durum wheat flours (<180 μm) were milled on the experimental mill (Laboratory mill, Bühler MLU-202, Switzerland) with six grinding passages, three fluted roll break and three smooth roll reduction passages. The characteristics of the obtained flour correspond to those of flour with 0.45 to 0.55% of ash.

A basic bread formula, based on the flour weight, was used, as follows: comprised flour (300g), yeast (7.5 g), salt (6.0 g), vegetable fat (3.0 g) and water. The ingredients were mixed for 5 minutes in a laboratory Diosna mixer (Dierks & Söhne maschinenfabrik, Osnabrück, Germany), and the dough was left for ripening (40 min) at min 75% relative humidity and 30±1°C. Then, the dough was divided into 115±1 g portions, manually rounded, rolled and put into tin pans (9.5 × 7.5 cm × 5.5 cm). The final fermentation lasted 43 min. The breads were baked at 230°C for 20 min in a deck type oven (Miwe condo, D-97450, Arnstein, Germany). All baking experiments were performed in

four repetitions. Baked breads were chilled and stored at 24°C and relative humidity of 34% for 24 h and then bread quality was evaluated.

Bread loaf volumes (mL) and specific volumes (mL/g) were determined by the VolScan profiler (Stable Microsystem, Surrey, UK). The hardness, chewiness, cohesiveness, resilience and springiness of bread crumbs were instrumentally recorded on the TA.XTplus texture analyser (Stable Micro Systems, England, UK) equipped with a 36-mm cylindrical probe according to the AACC method 7410A modified as described by FILIPČEV *et al*. (2010).

The sensory properties included the crumb elasticity and appearance of crumb pores (structure and uniformity, Dallman pores), as well as bread shape, smell, taste, aroma and chewiness. Twenty-four hours post baking, the breads were sliced and evaluated on a 5 point intensity scale by a 6-member panel with members trained in the sensory evaluation. Sensory properties of bread samples were evaluated by the 5-point category scale with end-points labelled from 1 to 5 as described by FILIPČEV *et al*. (2010).

The results were statistically analysed using Statistica software version 5.0 (StatSoft Co., Tulsa, OK, USA). Significance of differences between samples was analysed by the Tukey's test (HSD). Differences with $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Usually good baking quality refers to high loaf volumes, fine crumb structure with a large number of small sizes, thin-walled cells and soft texture. The relationship between crumb structure and crumb appearance may be self-evident, but this same crumb structure is also a determinant of the loaf volume, the resilience of the loaf, the crumb firmness, cohesiveness, chewiness and springiness. The results of the texture analysis of common and durum wheat breads are presented in Table 1. These results indicate statistically significant differences between the bread samples made from common and durum wheat genotypes in terms of their volume, specific volume, firmness and chewiness. The results obtained by the TPA analysis indicate better textural characteristics of the bread samples made from common and durum wheat grown in the 2010 season than of those grown in the following year. The loaf volume of the durum breads ranged from 189.5 to 228.1 ml and 160.1 to 165.1 ml in the 2010 and 2011 growing season, respectively. The loaf volumes of durum wheat bread samples with the best performance were significantly lower than the loaf volume of common wheat genotypes. These results are in agreement with results previously gained by other authors (EDWARDS *et al*., 2001; RAO *et al*., 2001).

Specific loaf volumes of bread samples made from common wheat genotypes cultivated in the 2010 season ranged from 3.3 to 3.5 ml/g, while specific the loaf volume were decreased by 36.4 and 45.7% during the following season (Table 1). Quite similar values

of specific volumes of breads made from spelt wheat flour (2.34-3.24 ml/g) and from rice flour (3.86 ml/g) were reported by FILIPČEV *et al*. (2013) and TSAI *et al*. (2012), respectively. Breads prepared from durum flours exhibited a significantly lower specific loaf volume. These results were expected since durum wheat is traditionally used in flat breads and specialty breads. Since the bread volume directly influences the firmness of bread, bread samples made from bread and durum wheat genotypes cultivated in the 2011 season with the lowest specific loaf volume had the highest bread crumb firmness and chewiness. Generally, the high bread crumb firmness was exhibited by all wheat genotypes during the first year, but significantly worsened during the second year.

Table1. Texture properties of common and durum wheat bread samples

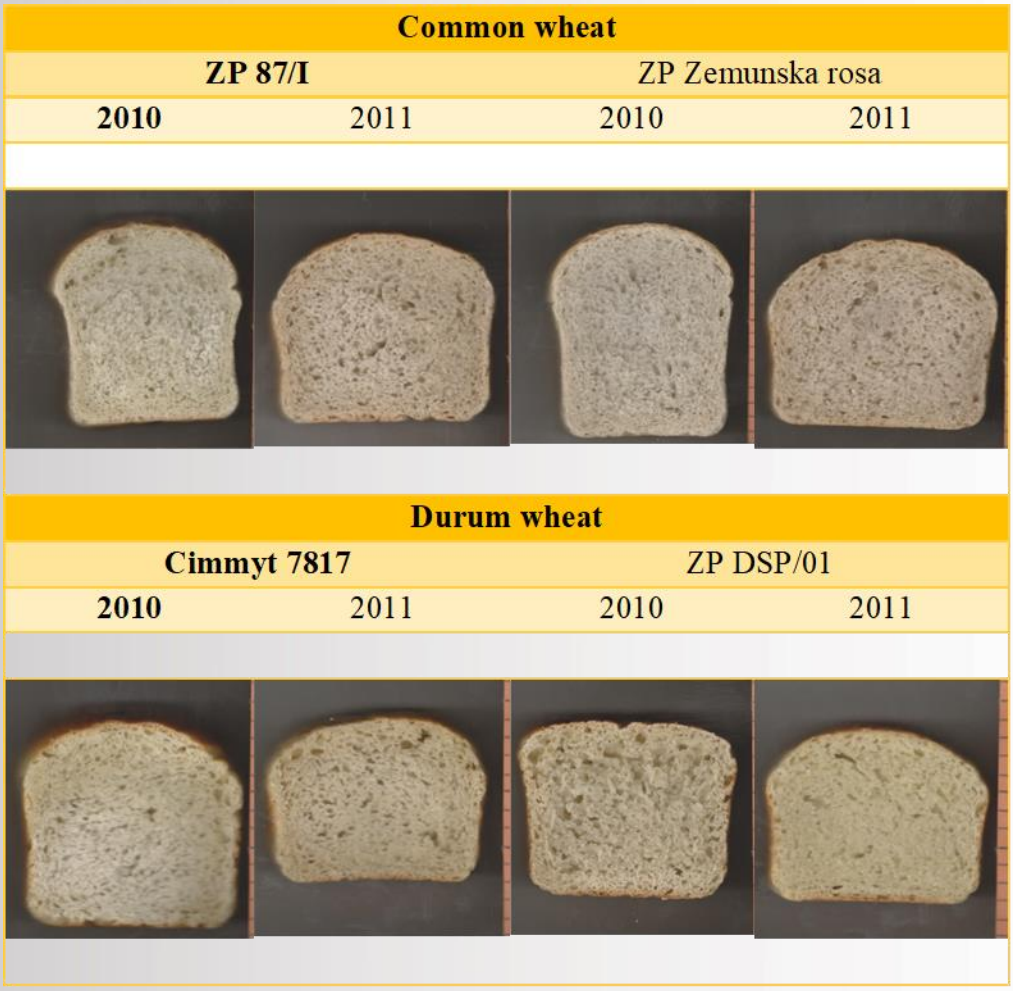
	Bread wheat					Durum wheat		
Sample	**ZP 87/I**		**ZP Zemunska rosa**		**Cimmyt 7817**		**ZP DSP/01**	
	2010	**2011**	**2010**	**2011**	**2010**	**2011**	**2010**	**2011**
Volume(ml)	305,0^{b}	201,2^{d}	327,5^{a}	186,9^{e}	228,1^{c}	160,1^{f}	189,5^{e}	165,1^{f}
Specific volume (ml/g)	3,3^{b}	2,1^{d}	3,5^{a}	1,9^{e}	2,4^{c}	1,7^{f}	2,1^{d}	1,7^{f}
Firmness(g)	538,3^{d}	1492,6^{b}	395,6^{d}	2132,6^{a}	1171,6^{c}	2230,4^{a}	1015,9^{c}	2083,3^{a}
Resilience	0,934^{ab}	0,922^{ab}	0,947^{a}	0,902^{b}	0,768^{d}	0,927^{ab}	0,542^{e}	0,820^{c}
Cohesiveness	0,528^{bc}	0,512^{cd}	0,549^{b}	0,477^{de}	0,463^{e}	0,603^{a}	0,385^{f}	0,518^{bc}
Chewiness(g)	265,7^{de}	705,2^{c}	213,3^{e}	919,9^{b}	416,0^{d}	1244,8^{a}	211,3^{e}	883,5^{b}
Springiness (%)	0,206^{de}	0,236^{c}	0,217^{e}	0,222^{b}	0,168^{d}	0,304^{a}	0,133^{e}	0,222^{b}

Means followed by the same letter within the same row are not significantly different ($p > 0.05$). Letters correspond to ranking of groups after the Tukey's test.

A bread made from the genotype ZP Zemunska rosa grown in the 2010 season had the smallest crumb firmness (395.6 g), while the bread made from the same genotype cultivated during 2011 had 5.4 fold firmer bread crumb. Soft crumb firmness caused the crumb structure of breads to have only few big pores of an irregular shape, uniformly distributed pores and the smooth crumb (Picture 1).

The texture profile analysis showed that the resilience and cohesiveness of common wheat genotypes during two years were not significantly different. However, resilience and cohesiveness were 1.4-fold higher in durum wheat genotypes cultivated in the 2010 season than in the second year.

Picture 1. Cross-section of common and durum wheat bread samples

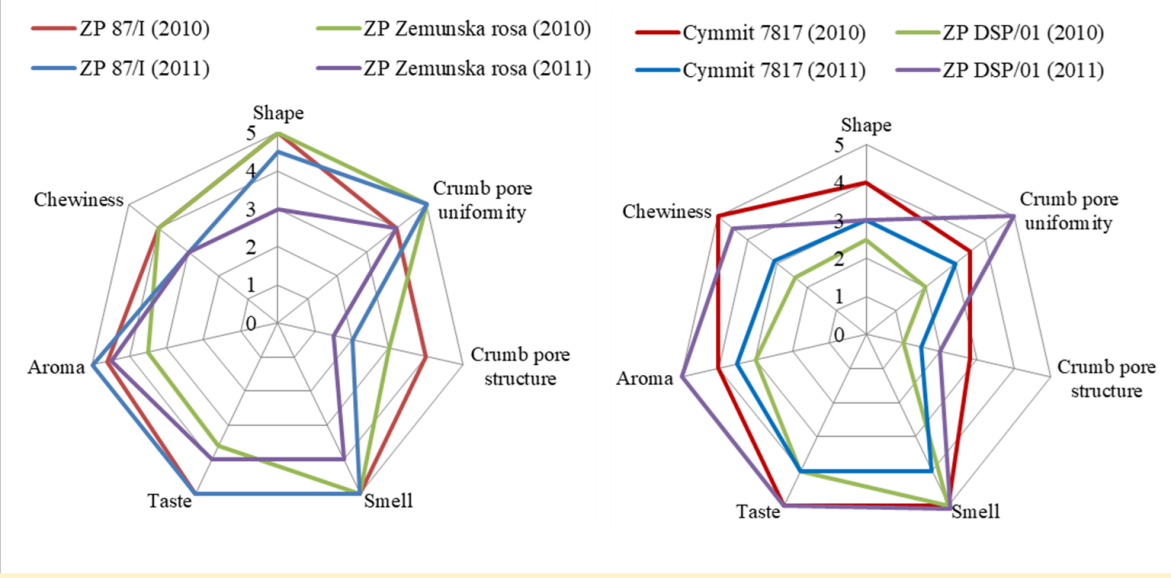


Springiness is associated with a fresh and elastic product (McCARTHY DF *et al*., 2005). Therefore, bread made from the durum genotype Cimmyt 7817 with the highest springiness value can be rated as high quality bread. However, bread samples of the durum genotype ZP DSP/01 had the lowest springiness, which, according to McCARTHY DF *et al.* (2005) is indicative of brittleness that reflects the tendency of the bread to crumble when sliced. TPA results showed that the 2011 common wheat genotypes exhibited a tendency towards the better crumb springiness by 1 and 12%, whereas durum wheat genotypes gave 40% and 45% better crumb springiness (Table 1, Picture 1).

Chewiness is the most indicative characteristic of bread. It is calculated by multiplying hardness, cohesiveness and springiness (ABDELGHAFOR *et al.*, 2011). Bread crumb chewiness had 2.7 and 4.3-fold and 3 and 4.2-fold higher values in the second experimental year for common and durum wheat genotypes, respectively. Bread samples made from the common wheat genotype ZP 87/I (213.3g) and the durum wheat genotype DSP/01 (211.3g) had a smallest chewiness, whereas bread samples made from the durum genotype Cimmyt 7817 (season 2011) had the greatest chewiness (1244.8 g).

Sensory properties of bread and durum wheat breads

Consumers have been increasingly demanding bread of high quality, with prolonged freshness, nutritional and sensory properties, but sensory perception still plays the most important role in consumer's preference and purchase of bread. Sensory properties of breads were determined 24 h after baking and results are shown in Graph 1 a and b.



a) b)

Graph 1. Spider web diagram of the sensory evaluation of breads:
a) common wheat (2010 and 2011), b) durum wheat (2010 and 2011).

Results of the sensory evaluation (Graph 1) revealed that the sensory properties (shape, crumb pore uniformity and structure, smell, taste, aroma and chewiness) of tested breads made from common and durum wheat genotypes cultivated in the 2010 season were overall better than that breads made from same wheat genotypes cultivated in the 2011 season.

Having in mind the individual sensory properties of the tested breads obtained from the common and durum wheat genotypes, it was noticeable that the greatest differences were expressed in terms of the bread shape, pore uniformity and the structure. This was expected since common wheat and durum wheat genotypes have quite different gluten protein properties. Bread samples made from common wheat genotypes grown in 2010 had uniform and quite uniform pores, while bread samples made from durum wheat genotypes cultivated in the same year had quite non-uniformly

and non-uniformly distributed pores, with the exception of the genotype ZP DSP/01 that had uniform pores. These results might be explained by the fact that the weak gluten matrix in durum wheat resulted in a discontinuous gluten structure and a low amount of gas generation in the dough. Therefore, the bread crumb could not keep sufficient gas during bread making, which affected the appearance of poorer texture properties of bread with non-uniform crumb pores.

Aroma is a sensory property that plays an important role in consumer's preference and purchase of bakery products. The formation of aroma compounds in bread crumb is highly influenced by the fermentation temperature, fermentation time and the yeast level (BIRCH *et al.*, 2013). In the crust, the volatile fraction is formed by thermal reactions occurring during baking, such as the non-enzymatic Maillard reactions (including Strecker degradation of carbonyl compounds) and caramelisation of sugars (BIANCHI *et al*., 2008; CHO and PETERSON, 2010).

Bread samples made from common (ZP 87/I) and durum (ZP DSP/01) wheat genotypes grown in 2011 had the best smell, taste and aroma, while the lowest score for aroma was established in the bread sample made from the wheat genotype ZP DSP/01 grown in 2010.

The sensory evaluation showed the greatest bread crumb chewiness for durum bread sample Cimmyt 7817 cultivated in 2010 (5), while chewiness significantly decreased (3.1) in 2011 (Graph 1b). The results of the texture analysis showed that the bread made from this genotype cultivated in 2010 had a low chewiness (416.0 g) and a high loaf volume (228.1 ml). On the other hand, the loaf volume showed reduction of 29.8% for the same genotype grown in 2011, while resistance to chewiness was higher by 300%. The comparison of the results gained by the texture analysis (Table 1) with the results obtained by the sensory analysis (Graph 1) shows that the sensory analysis did not correlate with the instrumental parameters.

CONCLUSION

Results of the sensory evaluation revealed that the sensory properties (shape, crumb pore uniformity and structure) varied greatly among the investigated bread samples made from common and durum wheat genotypes. All results show that the sensory evaluation of bread was a very difficult and sensitive task, often with contradictory results, even with the highly experienced and trained sensory panel. It can be concluded that investigated common and durum genotypes have quite different physical and sensory characteristics which could allow various possibilities of their use. Also, there is considerable interest in developing durum wheat suited to more general use for bread-making. Durum wheat with good baking quality is a desirable goal because such cultivars would have alternative markets in years of high production, by being used instead of common wheat, either singly or in blends with common wheat flour.

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REFERENCES

Abdelghafor, R.F., Mustafa, A.I., Ibrahim, A.M.H., Krishnan, P.G. (2011). Quality of Bread from Composite Flour of Sorghum and Hard White Winter Wheat. Advance Journal of Food Science and Technology, 3(1), 9-15.

Bianchi, F., Careri, M., Chiavaro, E., Musci, M., Vittadini, E. (2008), Gas chromatographic-mass spectrometric characterisation of the Italian Protected Designation of Origin 'Altamura' bread volatile profile. Food Chemistry, 110, 787–793.

Cho, I.H., Peterson, D.G. (2010). Chemistry of bread aroma: A review. Food Science and Biotechnology, 19, 575-582.

Edwards, N.M., Peressini, D., Dexter, J.E., Mulvaney, S.J. (2001). Viscoelastic properties of durum wheat and common wheat of different strengths. Rheologica Acta, 40, 142–153.

Filipčev, B., Lević, Lj., Bodroža-Solarov, M., Mišljenović, N., Koprivica, G. (2010). Quality characteristics and antioxidant properties of breads supplemented with sugar beet molasses-based ingredients. International Journal of Food Properties, 13, 1035–1053.

Filipčev, B., Šimurina, O., Bodroža-Solarov, M., Obreht, D. (2013). Comparison of the breadmaking performance of spelt varieties grown under organic conditions in the environment of northern Serbia and their responses to dough strengthening improvers. Hemijska Industrija, 67, 443-453.

McCarthy, D.F., Gallagher, E., Gormley, T.R., Schober, T.J., Arendt, E.K. (2005). Application of response surface methodology in the development of gluten-free bread. Cereal Chemistry, 82, 609–615.

Palumbo, M., Spina, A., Boggini, G. (2000). Durum Wheat Improvement in the Mediterranean Region, New Challenges, CIHEAM, Zaragoza, 515–518.

Rao, V.K., Mulvaney, S.J., Dexter, J.E., Edwards, N.M., Peressini, D. (2001). Stress-relaxation properties of mixograph semolina-water doughs from durum wheat cultivars of variable strength in relation to mixing characteristics and bread-making and pasta-making performance. Journal of Cereal Science, 34, 215-232.

Tsai, C.L., Sugiyama, J., Shibata, M., Kokawa, M., Fujita, K., Tasuta, M., Nabetani, H., Araki, T. (2012). Changes in the Texture and Viscoelastic Properties of bread containing Rice porrige during storage. Bioscience Biotechnology Biochemistry, 79, 331-335.

Veraverbeke, W. S., Delcour, J. A. (2002). Wheat protein composition and properties of wheat glutenin in relation to bread making functionality. Critical Reviews in Food Science and Nutrition, 42, 179-208.

BACTERIOPHAGES AND PHAGE-ENCODED DEPOLYMERASES

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ABSTRACT

Bacteriophages are the most numerous biological entities on the planet, occupying every habitat on Earth. Although they induce high mortality on bacterial realm, phages are seen as the driving forces of bacterial evolution and diversification. As natural killers of bacteria, their potential in treating bacterial infections was acknowledged immediately after their discovery a century ago. However, with the lack of understanding of phage biology and the emergence of antibiotics, the "phage therapy" was abandoned in the West. The perception changed with modern day rise of antibiotic resistance. Advantages of phage therapy include its potency against bacterial biofilms, structures underlining much of the reported resistance to antibiotics. Biofilm matrix consists of extracellular polymeric substances (EPS) which provide many advantages to bacterial cells especially in terms of resistance to various agents. But as with every other bacterial adaptation, phages evolved ways to subvert it and developed enzymes that degrade EPS polysaccharides, allowing them access to surface receptors. These enzymes, depolymerases, are phage-encoded and usually located on tail fibers of phage particles, but can also be released from bursting bacterial cells as soluble enzymes.

We isolated several phages active against multidrug-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, three members of WHO list of bacteria for which new antibiotics are urgently needed. Phages were isolated from Belgrade wastewaters, purified, screened for host range against laboratory collection and characterized based on plaque morphology. *A. baumannii* phages NOVI and ISTD produced halos around plaques on majority of sensitive strains, indicating presence of potent depolymerase(s).

Keywords: bacteriophages, depolymerases, multi-drug resistance, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*

INTRODUCTION

Drug-resistance in microbes and its associated mortality is heavily affecting global health security. For example, in 2019 more than 2.8 million antibiotic-resistant infections occurred in the United States, and more than 35,000 people died as a result.

Both numbers are considerably higher than the ones reported in 2013, triggering the U.S. Centers for Disease Control and Prevention (CDC) to declare entering the "post-antibiotic" era, and to prioritize bacteria regarding the level of concern (CDC report, 2019). Strategies for fighting this threat are mostly based on raising awareness of overuse and misuse of antibiotics, prevention of the spread of resistant infections, enhancing surveillance, early detection etc. However, no matter how extremely important the prevention and containment strategies are, morbidities and mortalities of multidrug-resistant bacterial infections dictate the need for therapeutics. The lack of new and efficient antibiotics revived the consideration of using bacteriophages, the natural predators of bacteria, as alternatives or adjuncts to antibiotic therapy. Bacteriophages were discovered and used to treat bacterial infections in the "pre-antibiotic" era, with first reports of human phage therapy as early as in 1917.

Among the most dangerous pathogens, that CDC prioritized as "urgent", are carbapenem-resistant *Acinetobacter* and *Enterobacteriaceae*. Genus *Acinetobacter* comprises several human pathogens, among which *A. baumannii* is the most important and clinically relevant species. It is characterized with great genome plasticity, which provides the host with the ability to easily acquire resistance determinants (HARDING *et al*., 2018). Carbapenem-resistant *Enterobacteriaceae* is a group of microorganisms notorious as "nightmare bacteria", since these pathogens present a major concern for patients in healthcare facilities (PEREZ *et al*., 2016). The group encompasses several genera including *Klebsiella* which, alongside *E. coli*, is responsible for most deaths claimed by the group.

In comparison with previously mentioned pathogens, *Pseudomonas aeruginosa* is the most studied and extensively reviewed species (LISTER *et al*., 2009; MORADALI *et al*., 2017). It is one of the pathogens burdening healthcare system for decades, and still presents one of the most serious threats (LAMBERT *et al*., 2011; CDC report 2019). Apart from being the leading nosocomial pathogen affecting hospitalized patients, it is also the main cause of morbidity and mortality in cystic fibrosis patients (MORADALI *et al*., 2017).

All mentioned pathogens can cause pneumonia as well as wound, bloodstream, and urinary tract infections. These infections tend to occur in patients in intensive care units, or with weakened immune systems. Further obstacle in combating all the mentioned bacteria is their biofilm forming ability. Biofilms are aggregates of bacteria immersed in self-produced extracellular polymeric substance (EPS) matrix consisting mostly of carbohydrates, but also of proteins, DNA and lipids. They aid bacteria in evasion of host immune system and antibiotic treatment, but also preserve bacteria in the environment, helping surface colonization, resistance to UV radiation, desiccation, etc. Capsular polysaccharides, that are anchored or otherwise linked to bacterial cells, can have an important role in biofilm formation (WANG *et al*., 2015), but may serve as target

molecules for bacteriophage attachment. Adsorption of some phages to capsules is mediated by enzymatic cleavage of exopolysaccharides that compose the capsule layers (SILVA *et al*., 2016). This activity is carried by depolymerases, enzymes usually located on phage tail fibers, but that can also be released from lysed cells as soluble proteins (PIRES *et al*., 2016).

In this work three clinical carbapenem-resistant strains of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* were used for phage isolation from Belgrade wastewaters, and total of six phages (two per strain) were isolated. All three bacterial strains were isolated from tertiary healthcare facilities and characterized earlier (NOVOVIC *et al*., 2015, NOVOVIC *et al*., 2017, JOVCIC *et al*., 2013). Phage plaque morphology, as well as host range against laboratory collection of clinical isolates was analyzed. Also, *A. baumannii* phage depolymerizing activity was investigated in more detail.

MATERIALS AND METHODS

Phage isolation and purification

Phages were isolated from two samples of Belgrade wastewaters by standard enrichment protocol and plaque assay (CLOKIE and KROPINSKI, 2009), using *A. baumannii* 6077/12, *K. pneumoniae* NI9 and *P. aeruginosa* MMA83 as host strains. After 4 rounds of plaque purification, single plaques were collected and further manipulated. Working phage suspensions were made by inoculation of single plaque into 10 ml of mid-exponential-phase culture of adequate host strains followed by overnight incubation. Obtained lysate was centrifuged and supernatant filtrated through 0.45 μm filters. For purification of phages from bacterial debris, CsCl density gradient ultracentrifugation was performed. Cultures of the appropriate hosts were grown to mid-exponential-phase in a volume of 500 ml and were infected with 1 ml of previously obtained phage suspension (titer 10^9 plaque forming units / ml) and incubated overnight. The obtained lysate was centrifuged, and supernatant collected and filtrated as previously described and treated with DNaseI and RNase A (Thermo Fisher) for 2 h at 37°C. Afterwards, NaCl and polyethylene glycol (PEG 8000, Sigma) were added (to obtain 1 M and 10% final concentrations, respectively), stirred and incubated overnight at +4°C to precipitate the phages. The precipitate was centrifuged at 13689.2 x g for 30 min at +4°C (Sorvall RC3B centrifuge, rotor GS3) and the pellet was resuspended in 4 ml of SM buffer. CsCl was added to reach 0.75 g/ml density. The samples were ultracentrifuged at 131980.8 x g for 24 h in Beckman Coulter Optima L-80 XP ultracentrifuge in rotor SW55 Ti, after which the distinct phage-containing bluish band was visible at the mid-point of the tube. The phages were extracted from tube by using a 22G needle and dialyzed overnight against 2 l of SM buffer at +4°C. Purified phages were filtered through 0.22 μm filters (Filtropur S 0.45, Sarstedt, Germany) and used in subsequent experiments.

Host range and depolymerase analysis

To determine the host range of isolated phages, as well as their possible depolymerase activity, phages were screened against bacteria from our laboratory collection. Total of 103 *A. baumannii*, 140 *K. pneumoniae*, and 32 *P. aeruginosa* strains were analyzed. All isolates were propagated in Luria-Bertani (LB) broth at 37°C with aeration (180 rpm), while stocks were made in LB supplemented with 15% (v/v) glycerol and kept at -80°C. The activity of phages and present depolymerases on the isolates from collection was tested using soft agar overlay spot assay. Briefly, 10 µl of overnight culture of each isolate was inoculated into 10 ml of melted soft agar. After vortexing, soft agar containing bacteria was overlaid on LB agar in Petri dish. Upon solidifying, 10 µl of phage suspension was spotted on top of the agar. After 24 h of incubation at 37°C, plates were inspected for appearance of clear growth inhibition zones surrounded by turbid halos. Isolates that were sensitive to phages were additionally inspected after 48, 72 and 96 h incubation at room temperature for detecting and tracking depolymerase activity.

The ability of phages to form halos on pre-grown bacteria was also analyzed. Bacteria were added to melted soft agar, poured over LB agar plates, and grown overnight. The following day, 10 µl of phage suspension was dropped on the surface of the lawn, the plate was incubated at 37°for additional 24 h, after which the plates were inspected for zone or halo formation.

RESULTS AND DISCUSSION

Plaque morphology

All phages were isolated upon forming clear translucent plaques inside lawns of susceptible bacteria. Phages NOVI and ISTD were isolated using *A. baumannii* 6077/12, phages LASTA and SJM3using *K. pneumoniae* NI9, while PLST and PSJM were active on *P. aeruginosa* MMA83 strain. Both *Acinetobacter* phages produced small (~1 mm diameter) plaques, which was also the case with both *Klebsiella* phages and *Pseudomonas* phage PSJM. On the contrary, phage PLST produced large, bulky plaques of up to 5 mm in diameter, suggesting highly efficient lytic nature of the phage, such as short and effective adsorption, short latent period and large burst size (Figure 1).

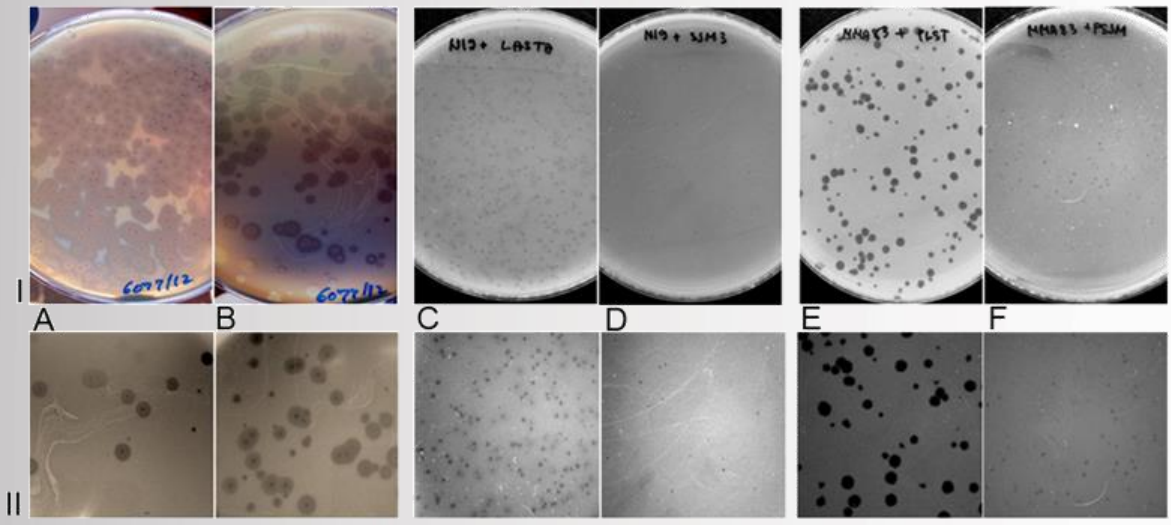

Figure 1. Plaque morphology. I Long shot and II close-up of: A) Phage NOVI, B) Phage ISTD, C) Phage LASTA, D) Phage SAJAM, E) Phage PLST, F) Phage PSJM. Note the large halos around plaques of NOVI and ISTD and small halos around plaques of LASTA.

Both *A. baumannii* phages NOVI and ISTD produced plaques that were surrounded by large (5-6 mm) halos (Figure 1A). With prolonged incubation at room temperature halos produced by phage ISTD were constantly enlarging 2-3 mm per day (Figure 2); the same results were obtained using phage NOVI (data not shown). Although more transparent and faintly noticeable, *K. pneumoniae* phage LASTA also produced halo. Unlike with NOVI and ISTD, it did not enlarge with time (data not shown), suggesting lack of enzyme diffusion through the agar.

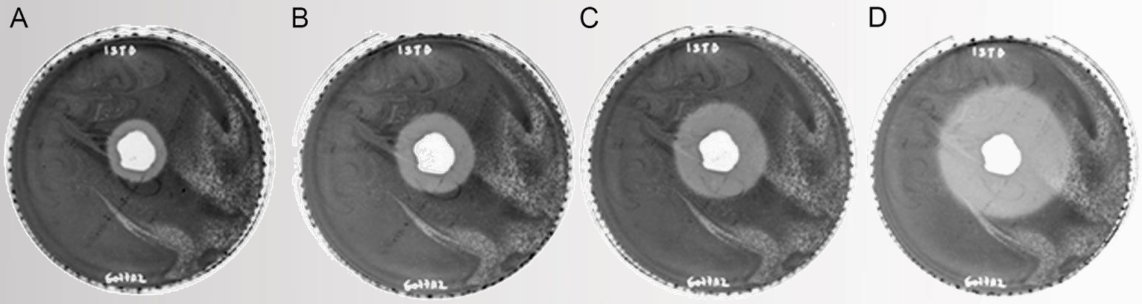

Figure 2. Spot assay of phage ISTD. A-D: 24, 48, 72 and 96 h after spotting the phages. Note the "growing" of the halo with time.

Observed concentric "growth" of halos formed around the plaques presents a visible manifestation of depolymerase-mediated cleavage of bacterial surface polysaccharides (LATKA *et al*., 2017). To test whether depolymerases can yield the same effect, without the paralleled phage-induced cell lysis and progeny release, similar test was performed, but on pre-grown bacterial cells. Namely, phages were applied 24 h later than in the first setting, giving bacteria time to reach stationary growth phase before the interaction. The results, although less apparent and without growth inhibition zone formation, were essentially the same as can be seen in Figure 3.

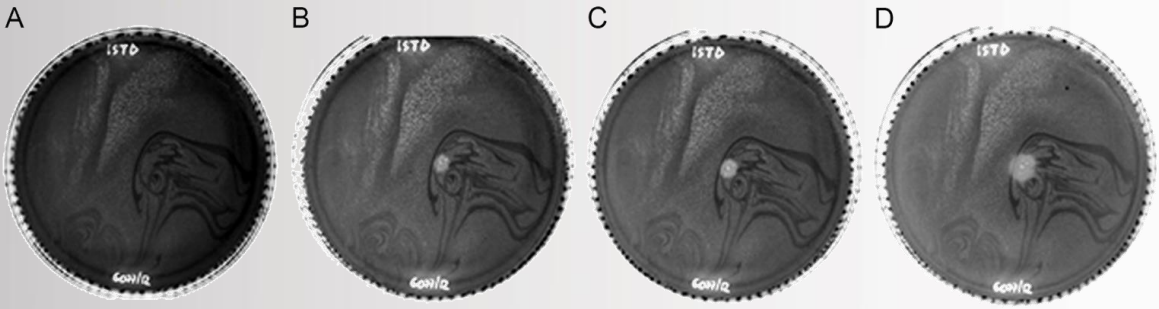

Figure 3. Spot assay of phage ISTD over overgrown lawn of bacteria. A: Nontreated lawn of bacteria, B-D: 24, 48 and 72 h after spotting the phages

Stationary phase bacteria are usually not susceptible to phage attack and infection, given the lack of available nutrients and slower metabolic rate of the host. Hence, only the enzymes already present in the phage suspension produced the enlargement effect. These results corroborated the notion that depolymerases also existed as free molecules in phage suspension. This suggests that depolymerase enzyme molecules leave phage-infected bursting bacterial cells both as virion-attached and as free molecules, capable of diffusing through the agar.

Host range analyses

To assess the applicative potential of isolated phages, we analyzed susceptibility of all available isolates from our laboratory collection to the isolated phages. Results are summed up in the Table 1.

Table 1. Host range of the isolated phages

Phage	Bacterial host	Host range	Number of isolates analyzed
NOVI	*A. baumannii*	36%	103
ISTD	*A. baumannii*	22%	
LASTA	*K. pneumoniae*	3.5%	140
SJM	*K. pneumoniae*	3.5%	
PLST	*P. aeruginosa*	81%	32
PSJM	*P. aeruginosa*	90%	

Although relatively high number of isolates was screened, determination of their genetic relatedness is needed to determine how many strains they actually represent. Still, given the number of isolates, especially in the cases of *A. baumannii* and *K. pneumoniae*, as well as the fact that those isolates originate from various hospitals and

have been collected for several years, it is reasonable to assume that obtained results adequately reflect the host range of the analyzed phages. Taking all into account, results presented suggest that *A. baumannii* phages NOVI and ISTD display moderately broad host range, *K. pneumoniae* phages LASTA and SAJAM have a very narrow range, while *P. aeruginosa* phages PLST and PSJM possess an exceptionally high host range, which remains to be confirmed with future testing.

CONCLUSION

In conclusion, new approaches to treating bacterial infections are growingly needed. Bacteriophages are one of the most promising solutions that may aid antibiotics in combat against multi-drug resistant infections. Several recent case studies, where phage application saved lives, moved phages into a scientific spotlight, as it became clear that they represent promising candidates for future therapeutics. In addition to lysing the bacterial cells, some phages are equipped with enzymes that can degrade bacterial exopolysaccharides. These phages can provide the edge advantage in overcoming biofilm infections, helping the action of antibiotics and immune system by eliminating at least a portion of infecting bacteria and degrading the protective matrix. Depolymerases of phages NOVI, ISTD and to lesser extent LASTA, demonstrated high activity independent of phage-bacteria interaction, and will be the object of further investigation.

One of the perspectives in phage therapy is forming and organizing phage banks, i.e. cataloging and characterizing all available phages. The eventual phage therapy approaches will rely on phage banks and phage cocktails. One of the main phage traits in this sense is their host range – the broader the host range of a phage, the better candidate it is for cocktail formulation and application. Phages described here, especially those active on *A. baumannii* and *P. aeruginosa,* fit well in this perspective and present good candidates for phage therapy.

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REFERENCES

CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019. https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf

Clokie M. R. J., Kropinski A. M. (2009). Bacteriophages: methods and protocols. Humana press, New York, NY, USA

Harding C. M., Hennon S. W., and Feldman M. F. (2018). Uncovering the mechanisms of *Acinetobacter baumannii* virulence. Nat. Rev. Microbiol. 16, 91–102.

Jovcic B., Lepsanovic Z, Begovic J., Rakonjac B., Perovanović J., Topisirović Lj., Kojic M. (2013). The Clinical Isolate Pseudomonas aeruginosa MMA83 Carries Two Copies of the blaNDM-1 Gene in a Novel Genetic Context. Antimicrob. Agents Chemother. 57(7): 3405–3407

Lambert M.L., Suetens C., Savey A., Palomar M., Hiesmayr M., Morales I., Agodi A., Frank U., Mertens K., Schumacher M., Wolkewitz M. (2011). Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to European intensive-care units: a cohort study. 11: 30–38.

Latka, A., Maciejewska, B., Majkowska-Skrobek, G., Briers, Y., and Drulis-Kawa, Z. (2017). Bacteriophage-encoded virion-associated enzymes to overcome the carbohydrate barriers during the infection process. Appl. Microbiol. Biotechnol. 101, 3103–3119.

Lister P., Wolter D., Hanson N. (2009). Antibacterial-resistant *Pseudomonas Aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. Clin. Microbiol. Rev. 22(4):582-610.

Moradali M., Ghods S., Rehm B. (2017). Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front. Cell Infect. Microbiol. 7:39. doi: 10.3389/fcimb.2017.00039

Novovic K., Mihajlovic S., Vasiljevic Z., Filipic B., Begovic J., Jovcic B. (2015). Carbapenem-resistant *Acinetobacter baumannii* from Serbia: Revision of CarO classification. PLoS One 10, 1–16. doi:10.1371/journal.pone.0122793.

Novovic K., Trudic A., Brkic S., Vasiljevic Z., Kojic M., Cirkovic I., Jovcic B. (2017). Molecular epidemiology of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* in Serbia from 2013-2016. Antimicrob. Agents Chemother. 61(5): e02550-16. https://doi.org/10.1128/AAC.02550-16

Perez F., Chakhtoura N., Papp-Wallace K., Wilson B., Bonomo R. (2016). Treatment Options for Infections Caused by Carbapenem-resistant Enterobacteriaceae: Can We Apply “Precision Medicine” to Antimicrobial Chemotherapy? Expert. Opin. Pharmacother. 17(6): 761–781.

Pires D., Oliveira H., Melo L., Sillankorva S., Azeredo J. (2016). Bacteriophage-encoded Depolymerases: Their Diversity and Biotechnological Applications. Appl. Microbiol. Biotechnol. 100(5):2141-51

Silva J., Storms Z., Sauvageau D. (2016). Host receptors for bacteriophage adsorption. FEMS Microbiology Letters. 363 (4): fnw002. doi: 10.1093/femsle/fnw002

Wang H., Wilksch J., Strugnell R., Gee M. (2015). Role of Capsular Polysaccharides in Biofilm Formation: An AFM Nanomechanics Study. ACS Appl. Mater. Interfaces. 7(23): 13007–13013.

EXPRESSION OF PROINFLAMMATORY CYTOKINES IN PREGNANT WOMEN WITH GESTATIONAL DIABETES MELLITUS

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ABSTRACT

Gestational diabetes mellitus (GDM) is one of the most common pathological conditions that can develop during pregnancy and is associated with high risk of health complications and adverse outcomes for both the mother and the fetus. It is also well known that gestational diabetes is characterized by increased inflammation. However, the implication of inflammatory cytokines in GDM pathophysiology remains unclear. The aim of the present study was to determine whether there are differences in the expression on RNA level of proinflammatory cytokines: TNF-α, IL-1β, IL-6 and IL-17 between GDM and normal pregnancies. This pilot study included 15 GDM patients and 15 healthy pregnant women (control group, CG). RNA was extracted from leukocytes, and real-time PCR was used to analyze relative gene expression of the examined cytokines. The results showed a statistically significant increase of IL-6 and IL-17 expression in GDM compared to controls ($p=0.016$ and $p=0.036$, respectively). There were no statistically significant differences between expression levels of TNF-α and IL-1β in GDM and CG groups ($p>0.05$). Our study points to a possible role of IL-6 and IL-17 in the pathophysiology of GDM. However, more research is needed for better understanding of inflammatory mechanisms underlying GDM. This is of vital importance for the development of preventive strategies and optimal management of GDM.

Keywords: gestational diabetes mellitus, proinflammatory cytokines, IL-6, IL-17

INTRODUCTION

Gestational diabetes mellitus (GDM) is one of the most common pregnancy complications with a frequency of up to 14% in women with live births (International Diabetes Federation, 2017). Even though it is present only during pregnancy and usually retreats several weeks following delivery, it is associated with high risk of health complications and adverse outcomes for both the mother and the fetus (VERMA *et al*, 2002; RETNAKARAN *et al*, 2010; OSTLUND *et al*, 2003; MITANCHEZ *et al*, 2015).

Previous studies have demonstrated that GDM is characterized by elevated oxidative stress and increased low-grade inflammation. We have receantly shown that GDM induces elevated oxidative stress and DNA damage (TOLJIC *et al*, 2017). Further, some proinflammatory cytokines, such as TNF-α and IL-6, have been found to play a role in insulin signaling pathways and GDM (BRIANA *et al*, 2009). Previous results have also pointed to the link between IL-1β and both diabetes mellitus type 1 (DM1) and type 2 (DM2) (DONATH *et al*, 2003). IL-17 have been suggested to have a role in the pathogenesis of pregnancy related complications such as gestational diabetes mellitus and preeclampsia (PE) (CAO *et al*, 2018).

To date, association between proinflammatory cytokines and GDM have not been fully elucidated. Therefore, the pathogenesis of GDM still remains unclear. The inflammation and oxidative stress biomarkers could give additional information on patient's risk for developing complications later in their life, but also can be used as potential therapy targets (OMU, 2013). To date, there is no screening test for GDM that could be done before clinical symptoms onset. Better understanding of GDM pathophysiology could have a crucial role in developing usefull screening test for early detection of women at risk of having GDM.

The aim of this pilot study was to investigate weather gestational diabetes mellitus is associated with elevated expression on RNA level of proinflammatory cytokines: TNF-α, IL-1β, IL-6 and IL-17.

MATERIALS AND METHODS

This pilot study was conducted at the Gynecology and Obstetrics Clinic "Narodni front" and included 30 pregnant women. According to their clinical features they were assigned to 2 groups: (1) 15 patients with GDM, (2) 15 healthy pregnant women with uncomplicated pregnancy (control group, CG). Diagnosis of GDM was based on criteria given by the American Diabetes Association (2003).

All patients signed an informed consent before they were included in the study. Peripheral blood samples, lifestyle information, medical and obstetrics details were collected between the 24th and the 36th gestational weeks. Exclusion criteria were multifetal pregnancy, fetal anomaly, fetal death, conditions that require termination of pregnancy, maternal diabetes history, any chronic or systemic diseases of the mother,

intolerance at <20 weeks of gestation and inability to give informed consent. The Ethic Committee of the Gynecology and Obstetrics Clinic "Narodni front" approved the study.

Peripheral blood samples were collected in EDTA vials and immediately centrifuged (3600 RPM) in order to separate buffy coat for RNA extraction and further analysis. Leukocytes total RNA was extracted from buffy coat using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) as it was previously described by manufacturer. Isolated RNA was stored at -80 °C. After that, 1.5 μg of total RNA and oligo (dT) primers were used for cDNA synthesis (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Waltham, MA). IL-1β, TNF-α, IL-6 and IL-17 corresponding cDNA were amplified using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Rox) (Solis BioDyne, Tartu, Estonia) kit and Line gene K fluorescence quantitative PCR detection system (BIOER Technology Co, Hangzhou, China). All experiments were done in duplicate. Quantitative PCR conditions included: initial DNA denaturation (10 minutes at 95°C), 45 cycles (30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C) and final extension (10 minutes at 72°C). Normalization was done using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control.

Relative expression levels were calculated using the $2^{-\Delta Ct}$ method. The results were analyzed using IBM SPSS Statistics and presented as arithmetical means with standard deviations (mean ± SD). Mann-Whitney *U* test was used for comparison of the results between groups. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This pilot study included 30 pregnant women (15 GDM patients and 15 healthy pregnant women). Figures 1 to 4 show average expression levels of proinflammatory cytokines in the examined groups. As our results showed, women with GDM had significantly higher expression of IL-6 compared to normal pregnancy women ($p=0.016$). In addition, relative expression of IL-17 significantly differed between the two groups, with higher expression in GDM patients ($p=0.036$). On the other hand, there was no statistically significant difference between expression levels of TNF-α and IL-1β in GDM and CG groups ($p>0.05$).

As one of the most common complications that can develop during pregnancy, GDM draws a lot of attention. Multiple studies have investigated the GDM pathophysiology, but exact mechanisms underlying this pregnancy complication remain unclear. Our group already showed that GDM leads to the increase of not only oxidative stress, but also DNA damage and chromosomal aberrations (TOLJIC *et al*, 2017.). This pilot study attempted to shed light on another aspect of GDM in order to get a better picture of GDM pathophysiology, which is of vital importance for a better understanding of this pathological condition and for the optimization of its management.

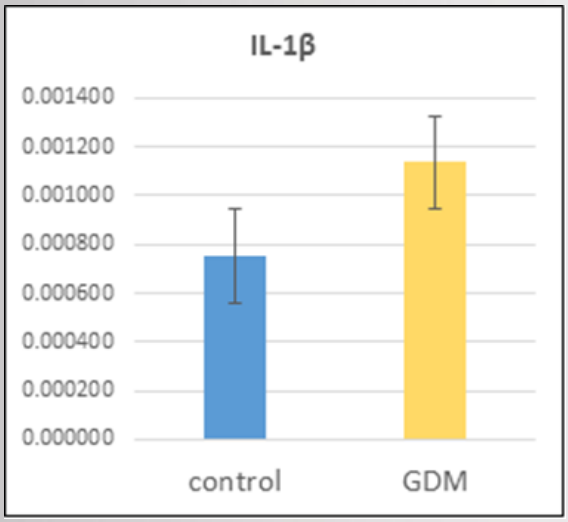

Figure 1. Average expression levels of IL-1β in CG and GDM group

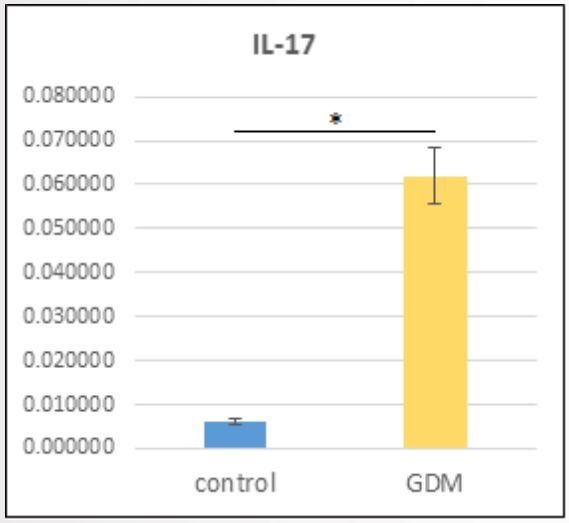

Figure 2. Average expression levels of IL-6 in CG and GDM group

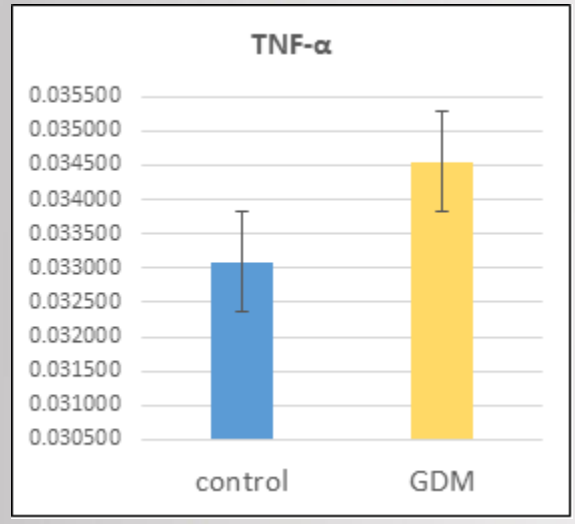

Figure 3. Average expression levels of TNF-α in CG and GDM group

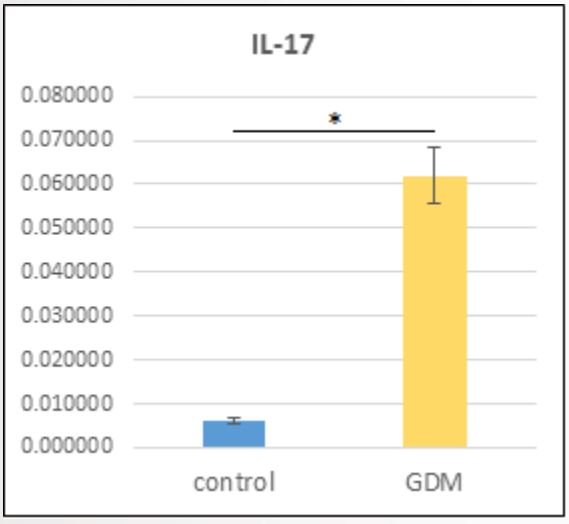

Figure 4. Average expression levels of IL-17 in CG and GDM group

It is well known that inflammation plays a role in GDM pathology. Our results indicate that pregnant women with GDM have higher IL-6 mRNA levels compared to controls. This is in concordance with the results of some other studies. MORISSET and his colleagues (2011) concluded that GDM is associated with higher IL-6 levels, both during and after pregnancy and that IL-6 expression increase is independent of obesity status. This group of authors gave two possible explanations on how IL-6 regulates insulin resistance in GDM: either elevated IL-6 generates insulin resistance in liver cells leading to global insulin resistance or (as shown by *in vitro* studies) IL-6 participates in the negative regulation of insulin signaling and glucose metabolism in adipocytes.

JOHNSON *et al*. (2002) demonstrated that preeclampsia (PE), another pregnancy-induced complication, was also accompanied by the augmentation of IL-6 expression. Interestingly, opposite to Johnson's and our results, SLJIVANCANIN JAKOVLJEVIC *et al*. (2019) reported that IL-1β and TNF-α were the main cytokines involved in the pathogenesis of PE in Serbian women.

Contrary to TNF-α and IL-6, as literature search revealed, the role of IL-17 in pregnancy complications has not been frequently investigated, yet. We showed that IL-17 mRNA has also a significantly higher expression in women with GDM than in healthy pregnant women. In line with our results, CAO and his group (2018) detected elevated expression of IL-17 in GDM and PE patients, as well. They suggested that IL-17/IL-35 imbalance could be involved in the development of endothelial dysfunction, which would in turn lead to elevated blood pressure and proteinuria in PE. The exact role of IL-17 in GDM pathophysiology still needs to be clarified.

Some studies proposed that IL-1β is correlated to DM1 and DM2 (DONATH *et al*, 2003) and MOJTABA and his colleagues (2011) assumed that higher levels of IL-1β are associated with pancreatic beta-cell dysfunction and reduced insulin secretion. However, our results showed no correlation between this cytokine and gestational diabetes mellitus.

TNF-α has been suggested to exert an inhibitory effect on insulin secretion in GDM, leading to further hyperglycemia (McLACHLAN *et al*, 2006). In the present study higher levels of TNF-α have been registered in GDM compared to normal pregnancies, but without reaching a statistically significant difference between the two groups, probably due to the small sample size. This is in line with the results of GAO *et al*. (2008) who also found increased levels of TNF-α in Chinese patients with GDM.

GOMES and his group (2013) previously pointed that new studies with well-defined, more stringent methodological parameters and better selection criteria are needed in this field. We agree that further research on larger case and control groups is required for better understanding of the role of proinflammatory cytokines in GDM.

CONCLUSION

GDM can have long-term consequences for both the mother and her infant. To date, GDM can be diagnosed only after the 20th gestational week, when clinical symptoms are already present as a consequence of pregnancy-induced hyperglycemia. Development of adequate screening tests that could be performed before clinical manifestation of GDM would prevent its negative outcomes. Understanding GDM pathophysiology is a prerequisite for the development of preventive strategies. Our study points to a possible role of two cytokines, IL-6 and IL-17, in GDM. Hence, the possibility of using cytokines as biomarker of pregnancy complications should be taken into consideration.

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REFERENCES

American Diabetes Association, Gestational diabetes mellitus, Diabetes Care 26 (2003). S103–S105.

Briana DD, Malamitsi-Puchner A. (2009). Reviews: adipocytokines in normal and complicated pregnancies. Reprod Sci. 16(10):921-37.

Cao W, Wang X, Chen T, Xu W, Feng F, Zhao S, Wang Z, Hu Y, Xie B (2018). Maternal lipids, BMI and IL-17/IL-35 imbalance in concurrent gestational diabetes mellitus and preeclampsia. Exp Ther Med. 16(1):427-435.

Donath MY, Størling J, Maedler K, Mandrup-Poulsen T (2003). Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes, Review. J Mol Med (Berl). 81(8):455-70.

Gao XL, Yang HX, Zhao Y (2008). Variations of tumor necrosis factor-alpha, leptin and adiponectin in mid-trimester of gestational diabetes mellitus. Chin Med J (Engl). 20;121(8):701-5.

Gomes CP, Torloni MR, Gueuvoghlanian-Silva BY, Alexandre SM, Mattar R, Daher S (2013). Cytokine levels in gestational diabetes mellitus: a systematic review of the literature, Review. Am J Reprod Immunol. 69(6):545-57.

International Diabetes Federation. IDF Diabetes Atlas. 8th ed. Brussels, Belgium: International Diabetes Federation; 2017. http://www.diabetesatlas.org

Johnson MR, Anim-Nyame N, Johnson P, Sooranna SR, Steer PJ (2002). Does endothelial cell activation occur with intrauterine growth restriction? BJOG. 109(7):836-9.

McLachlan KA, O'Neal D, Jenkins A, Alford FP (2006). Do adiponectin, TNFalpha, leptin and CRP relate to insulin resistance in pregnancy? Studies in women with and without gestational diabetes, during and after pregnancy. Diabetes Metab Res Rev. 22(2):131-8.

Mitanchez D, Yzydorczyk C, Siddeek B, Boubred F, Benahmed M, Simeoni U (2015). The offspring of the diabetic mother--short- and long-term implications. Best Pract Res Clin Obstet Gynaecol. 29(2):256-69.

Mojtaba E, Mahdi K, Mehdi KJR, Amir S (2011). Serum interleukin-1 beta plays an important role in insulin secretion in type II diabetic. International Journal of Biosciences, 1:3, 93-99.

Morisset AS, Dubé MC, Côté JA, Robitaille J, Weisnagel SJ, Tchernof A (2011). Circulating interleukin-6 concentrations during and after gestational diabetes mellitus. Acta Obstet Gynecol Scand. 90(5):524-30.

Omu EA (2013). Pro-inflammatory cytokines, lipid metabolism and inflammation in gestational diabetes mellitus as cause of insulin resistance. Chapter from the book Gestational Diabetes - Causes, Diagnosis and Treatment. 79-102.

Ostlund I, Hanson U, Björklund A, Hjertberg R, Eva N, Nordlander E, Swahn ML, Wager J (2003). Maternal and fetal outcomes if gestational impaired glucose tolerance is not treated. Diabetes Care. 26(7):2107-11.

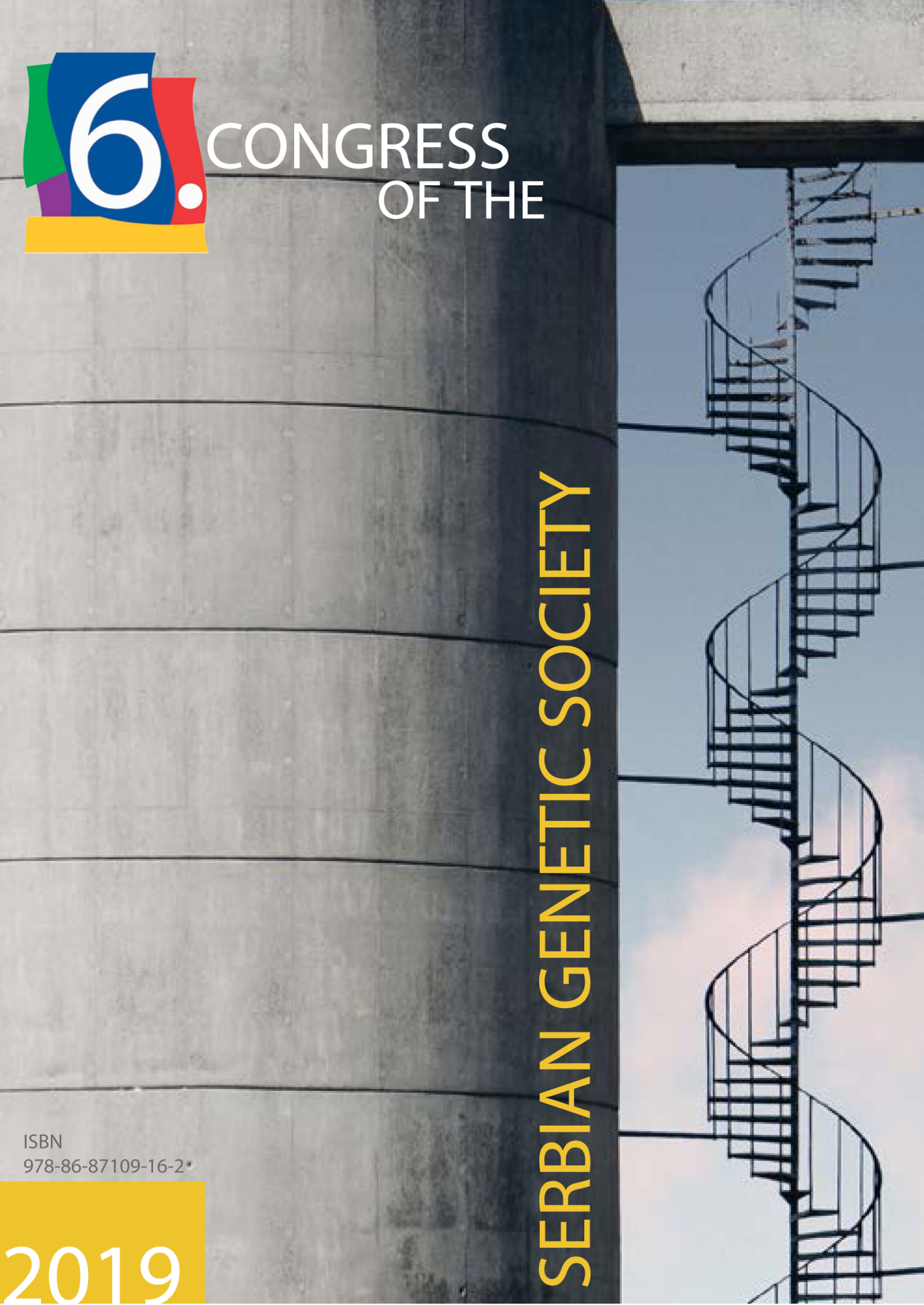
Retnakaran R, Qi Y, Connelly PW, Sermer M, Zinman B, Hanley AJ (2010). Glucose intolerance in pregnancy and postpartum risk of metabolic syndrome in young women. J Clin Endocrinol Metab. 95(2):670-7.

Sljivancanin Jakovljevic T, Kontic-Vucinic O, Nikolic N, Carkic J, Soldatovic I, Milasin J (2019). Glutathione-S-transferase M1 polymorphism and pro-inflammatory cytokines tumour necrosis factor-α and interleukin-1β are associated with preeclampsia in Serbian women. Am J Reprod Immunol. 81(5):e13105.

Toljic M, Egic A, Munjas J, Karadzov Orlic N, Milovanovic Z, Radenkovic A, Vuceljic J, Joksic I (2017). Increased oxidative stress and cytokinesis-block micronucleus cytome assay parameters in pregnant women with gestational diabetes mellitus and gestational arterial hypertension. Reprod Toxicol. 71:55-62.

Verma A, Boney CM, Tucker R, Vohr BR (2002). Insulin resistance syndrome in women with prior history of gestational diabetes mellitus. J Clin Endocrinol Metab. 87(7):3227-35.

6. CONGRESS OF THE SERBIAN GENETIC SOCIETY



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