UDK 630* 165 (001)
https://doi.org/10.31298/sl.147.7-8.5

Izvorni znanstveni članci – Original scientific papers
Šumarski list, 7–8 (2023): 363–374

ARE OXIDATIVE STRESS SCREENING TESTS ENOUGH FOR SMALL SCALE QUERCUS ROBUR GENETIC MONITORING?

JESU LI TESTOVI PRAĆENJA OKSIDACIJSKOG STRESA DOVOLJNI ZA GENETIČKI MONITORING HRASTA LUŽNJAKA U MALOM RAZMJERU?

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SUMMARY

Although being present much widely in terms of its geographical distribution, pedunculate oak (*Quercus robur* L.) represents one of the crucial species in forest ecosystems in South Eastern Europe. We wanted to contribute to existing efforts in designing genetic monitoring methodology for this noble broadleaved species, through the screening of genotypes with different health status and belonging to two phenological varieties [early (var. *praecox*) vs late (var. *tardissima*)] using seven oxidative stress parameters (total protein content, total phenolic compounds content, total flavonoids content, DPPH, ABTS, NO radical scavenger activities and Ferric reducing antioxidant power essay - FRAP). Results of this study provided significant biological message, contributing to better understanding of existing biodiversity of pedunculate oak in Serbia. Four out of seven oxidative stress screening tests (total phenolic compounds content, total flavonoids content, RSC NO and FRAP) clearly discriminated late vs early individuals sampled from selected pedunculate oak gene pool. Although presented results did not show significancy in using mentioned parameters to distinct damaged from vital individuals, nevertheless, knowledge gained through discussion and comparison with previous studies contributed to precise determination of biochemical tests which might be used in future efforts of genetic monitoring of this species.

KEY WORDS: antioxidative, biodiversity, ecotype, pedunculate oak, stress

INTRODUCTION

UVOD

Pedunculate oak (*Quercus robur* L.) is the dominant tree species in many natural and planted European lowland and riparian forests excluding large parts of Spain, Portugal and North Scandinavia (Sergeant et al., 2011). Among the deciduous, ring-porous oak species, pedunculate oak *Q. robur* is one of the most abundant species in Europe (Haneca et al., 2009). *Q. robur* is typically found in mesic sites across floodplains and valleys of large European rivers, lowlands and rocky sites (Madrigal-González et al., 2017).

According to RCP climate scenarios, pedunculate oak (*Q. robur* L.) has been marked as the most affected tree species due to ongoing, intensive climate change (de Wergifosse, 2022). The decline of oak forests can cause great economical losses since oaks are considered highly valuable tree

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species due to their long history of exploitation for wood production and construction (Mikac et al., 2018).

Q. robur has a high-water requirement and displays low water use efficiency, and is thus, sensitive to drought stress (Čehulić et al., 2019; Bose et al., 2021; Kostić et al., 2022). In Serbia, the majority of pedunculate oak stands are situated in the northern part of the country, along with the Sava and the Danube rivers, and in Central Serbia, along the Morava River (Trudić et al., 2013; Stojnić et al., 2014; Rađević et al., 2020). Despite the occurrence and dispersal, pedunculate oak in South Eastern Europe and Serbia alike is under strong influence of drought stress, coming from different natural sources. Climate change, as cumulative challenging factor, is a major driver of drought, and, therefore, influencing *Q. robur* gene pools in Serbia with dry seasons and shortage of water availability in soil and air (Kostić et al., 2019; 2021a, 2021b, 2022; Stojanović et al.,2021). Although a number of studies conducted in the Sava river basin (Medarević et al., 2009; Bauer et al., 2013; Stojanović et al., 2013; Stojnić et al., 2014; Stojanović et al., 2014; Kostić, 2019, 2021a, 2022) have been focused on different causes of oak mortality (attacks of pests and diseases, climate fluctuations, water level change, and inappropriate tending measures), there is still no consensus within the scientific community about the causes of oak dieback in south-east Europe (Stojanović et al., 2015, Mikac et al., 2018). Dependence of this species on soil and water properties plays an uncertain role in its survival and stress response (Kostić et al., 2021c) and, therefore, a focus on ecophysiology of this valuable broadleaved species should be a priority in the research, breeding and forest management (Pilipović et al., 2020).

There have been many studies done on this species in Europe and Serbia. Genetic variability of *Q. robur* has been widely studied (Barreneche et al., 1998). Namely, morphological characteristics (Kremer et al., 2002; Batos et al., 2006; Battos et al., 2017), biochemical markers (Streiff et al., 1998; Gömöry et al., 2001; Finkeldey and Hattemer, 2010) and molecular markers (Scotti-Saintagne et al., 2004; Muir and Schloetterer, 2005; Neophytou et al., 2010; Katičić Bogdan et al., 2018). In Serbia, genetic diversity of *Q. robur* was extensively researched, showing rich heterozygosity and conserved variability for decades now (Trudić et al., 2013; Trudić and Avramidou et al., 2021a; Kesić et al., 2021). Significance of the recent study of Trudić and Avramidou et al. (2021a) reflects in two aspects: it tackled genetic diversity of the same population used in this study and it indicated rich heterozygosity and stable genetic diversity. In addition, biochemical, physiological and morphological diversity of *Q. robur* from Serbia were assessed, under open, controlled and stressful conditions, tracking various biochemical and morphological traits and parameters (Stojnić et al., 2019; Pilipović et al., 2020; Vaštag et al., 2020; Vaštag et al., 2022, Kebert et al., 2022a, 2022b, 2022c). These studies gave an immense contribution to the understanding of genetical and physiological foundations of pedunculate oak diversity on the territory of Serbia, especially in terms of understanding possible links of used morphometrics and physiological markers to differ oak genetic structure and its drought stress resilience capacity. Selected markers in presented studies, alongside with biochemical parameters covered in this, are having strong genetical basis, and yet being facilitated by the environmental influence as well. Oxidative stress parameters are interlinked with secondary metabolism of the plant, which represents the part of the plant ontogenesis directly influenced by environmental conditions (Popović and Štajner, 2008). As it was suggested by previous studies (Popović et al., 2013; Stojnić et al., 2016; Pellegrini et al., 2019; Ghanbary et al., 2021; Dadkhah-Aghdash et al., 2022; Kebert et al., 2022b) on the phenolic compounds and antioxidant capacity of oak and other tree plants species, these biochemical parameters are proposed/monitored as important markers in selection and breeding of the species when tolerance and adaptation to (a)biotic stresses are concerned.

Due to its high intra-species and intra-provenance variations, pedunculate oak provides a valuable gene pool for the selection of superior, climate-resistant genetic material (Kostić et al., 2021). The phenologically different varieties growing in the Serbian lowland region have also been shown to differ regarding pathogen resistance, wood quality, phenology, morphology, chemical characteristics, etc. (Batos, 2012a; Batos, 2012b, Bobinac et al., 2012). Considering the importance of pedunculate oak for forest biodiversity and ecosystem restoration initiatives, constant efforts for its *ex situ* and *in situ* conservation through research of genetic diversity are of profound importance in order to mitigate climate change (Stojnić et al., 2019). Apart from these efforts, breeding of more resilient provenances across landscape presents one of the efficient ways for future adaptation of forest tree species to climate change, since adaptation enables plants to optimize their life processes in prevailing environmental conditions at an evolutionary scale. To achieve these long-term goals of pedunculate oak conservation and selection initiatives, integrating different research fields such as plant physiology, genetics and biochemistry presents a holistic, interdisciplinary solution that is appropriate for current and upcoming environmental challenges caused by climate change (Pilipović et al., 2020).

Taking into consideration that common biochemical parameters may contribute to understanding the underpinning plant defense mechanisms against occurring drought stress and, therefore, used as reliable adaptability descriptors in the context of climate change, the aim of this study was to determine if any of 7 proposed oxidative screening tests might be detrimental for long-term genetic monitoring of two varieties of pedunculate oak.

MATERIALS AND METHODS
MATERIJALI I METODE

Sampling – *Uzorkovanje*

In order to properly evaluate individuals for oxidative stress screening tests, we first selected them according to phenophase [early (var. *praecox*) vs late (var. *tardissima*)] and physiological groups (vital or damaged). The main criterion for determining the affinity of trees to one of the physiological status groups (vital or damaged) was the degree of crown damage, which was assessed according to Dubravac et al. (2011): trees with defoliation above 25 % were considered significantly damaged trees, while trees with canopy damage up to 25 % were considered undamaged or vital trees. Presence of damage caused by insect pests and pathogens, such as damage to stems caused by xylophagous and phloemophagous insects (e.g., *Scolytus* spp., *Agrilus* spp., *Cerambyx cerdo*, *Zeuzera pyrin*) (Marković and Stojanović, 2011; Mihajlović et al., 2015), and necrotic lesions on stems caused by *Diplodia seriata* (Zlatković et al., 2018) were used as an additional criterion in tree selection, especially in discrimination between vital and damaged trees. Crown condition was assessed in the same year as sampling. Leaf samples from 42 individuals [21 individuals of *Quercus robur* var. *praecox* (11 vital + 10 damaged) and 21 individuals of *Q. robur* var. *tardissima* (11 vital + 10 damaged)] were collected from seed stand located in the Northern part of Serbia (N 45° 00‘12.66”; E 019° 08‘50.4”) during springtime in April and May 2013. The altitude of the examined stand was ranging from 81 to 83 m above sea level (a.s.l.). The geological substrate was defined as alluvial sand sediments with mostly gley soils ranging from riparian black soil and black meadow soil to brown forest soil. Hydrological conditions were characterized by the absence of flooding and soil moisture was strongly dependent on fluctuations of groundwater table levels. Climate in the sampling area is defined as temperate continental to modified continental, with semi-humid and warm summers (Kottek et al., 2006).

Plant material – *Biljni material*

Phenological varieties [early (var. *praecox*) vs late (var. *tardissima*)] and physiological group (vital or damaged) were the observed variables in tested individuals of pooled early oak with vital (PEOV), pooled early oak with damaged (PEOD), pooled late oak with vital (PLOV) and pooled late oak with damaged (PLOD) canopies. Each pooled group comprised 10 or 11 samples in total, measured in triplicates in order to achieve optimal statistical significance for each group (for more, see Statistics sub-section). Collected leaves were immediately put and labeled on aluminum foliage, preserved on dry ice, and transported to the Laboratory for Chemical and Biochemical Analysis at the Institute of Lowland Forestry and Environment (ILFE) for further processing.

Oxidative stress screening tests – *Testovi praćenja oksidacijskog stresa*

Two grams of oak leaves were homogenized in a cooled mortar with pestle with 10 ml of phosphate buffer (0.1 M KH_2PO_4, pH=7), centrifuged for 10 min at 11000 rpm and supernatant was used for further oxidative stress screening test (Table 1), as per authors instructions. All analyses were determined spectrophotometrically in microwell-plates by Multiplate reader MultiScan GO (Thermo Scientific, Germany).

Statistics – *Statistika*

Values of the biochemical parameters were expressed as mean value ± standard error of determinations made in

Table 1. Oxidative stress screening tests used in this study.
Tablica 1. Testovi praćenja oksidacijskog stresa korišteni u ovoj studiji.

Number Broj	Test	Test Acronym Kratica za test	Units Jedinice	Reference Referenca
1	Total soluble protein content Ukupni sadržaj topivih bjelančevina	TSP	mg BSA/g	Bradford, 1976
2	Total phenolic compounds content by Folin-Ciocalteu method Ukupni sadržaj fenolnih komponenti određen Folin-Ciocalteu metodom	TP	mg GA g-1	Singleton et al., 1999
3	Total flavonoids content Ukupni sadržaj flavonoida	TF	mg of quercetin equivalents per g of fresh weight of plant material (mg QE/g FW)	Chang et al., 2002
4	ABTS-radical scavenger capacity ABTS – test kapaciteta hvatača radikala	ABTS	% of neutralized radicals [% RSC= 100 – (A blank- A sample) x 100/ A blank]	Arnao et al., 2001
5	DPPH-radical scavenger capacity DPPH – test kapaciteta hvatača radikala	DPPH	% of neutralized radicals [% RSC= 100 – (A blank- A sample) x 100/ A blank]	Soler-Rivas et al., 2000
6	NO• -radical scavenging capacity NO• – test kapaciteta hvatača radikala	RSCNO	% of neutralized radicals [% RSCNO= 1 – (A sample/A blank) x 100%]	Tsai et al., 2007
7	Ferric reducing antioxidant power-FRAP Esej antioksidativne moći redukcije gvožđa	FRAP	mg of Trolox equivalents per g of fresh weight of plant material (mg TEAC/g FW)	Benzie and Strain, 1999

triplicates and tested by two-way analysis of variance (ANOVA) followed by comparison of the mean values by Duncan's multiple range post-hoc test for $p<0.05$. In two-way ANOVA, phenological variety and physiological status were used as dependent variables, which were interpreted using the Fisher (F) test and their statistical significance levels (p). The R environment was used for all statistical data processing (R Core Team, 2013). The "*rstatix*" R package (Kassambara, 2020) was used to calculate descriptive statistics and run two-way ANOVA, "*ggfortify*" for PCA analyzes and "*corrplot*" for correlation matrix of Pearson's correlations. Finally, "*ggplot2*" R package was used for other data visual representations.

RESULTS
REZULTATI

Obtained results of biochemical parameters showed that leaves from late oak trees had higher protein and phenolic compounds contents than early oak trees, regardless of the physiological group (vital or damaged) (Table 2). Moreover, for the sake of better presentation of the results from Table 2, we have presented them graphically (Figure 1 and 2). PLOD and PLOV leaves had the highest, while PEOV and PEOD leaves had the lowest content of total flavonoids (Table 2).

As for radical scavenging capacity tests, all tested leaves had low scavenging capacity. ABTS- and NO- radical scavenger tests showed lower than 50% scavenging for all samples, while DPPH-test showed insignificantly higher than 50% scavenging for PEOS, PEOV, PLOV leaves (56.15%, 57.28% and 51.58%, respectively) and 41.69% for PLOD leaves. Late oak leaves had higher ferric reducing antioxidant power (203.5 and 180.4 mg TE/g FW, respectively) than early ones (93.9 and 85.5 mg TE/g FW, respectively), regardless of the physiological group (Table 2).

Two-way Anova was performed for phenological varieties [early (var. *praecox*) vs late (var. *tardissima*)], physiological groups (vital or damaged) and their interaction as dependent variables, with the aim to determine whether there was an influence of tested dependent variables on the variability of the analyzed parameters. According to obtained results, phenological variety has no influence on protein and DPPH– radical scavenger activity, while physiological group has no influence on total phenolics content and subsequently on FRAP, ABTS- and NO- ra-

Table 2. Proteins, total phenolic (TP) and flavonoids (TF) compounds content and radical scavenger capacity (ABTS, DPPH, NO, FRAP) of tested oak leaves. Each value is the mean value of all tested samples ± standard error ($\bar{X}\pm$Se). Results marked with different letter differ significantly at $p<0.05$ (Duncan's multiple range test).

Tablica 2. Sadržaj proteina, ukupnih fenolnih (TP) i flavonoidnih (TF) spojeva i sposobnost hvatanja radikala (ABTS, DPPH, NO, FRAP) testiranog lišća hrasta. Svaka vrijednost je srednja vrijednost svih ispitanih uzoraka ± standardna pogreška ($\bar{X}\pm$Se). Rezultati označeni drugim slovom značajno se razlikuju pri $p<0,05$ (Duncanov test višestrukog raspona).

Ecological form Ekološka forma	$\bar{X}\pm$Se						
	TSP (mg/g FW)	TP (mg GAE/g FW)	TF (mg QE/g FW)	ABTS – radical scavenger test (%) ABTS – test hvatača radikala	DPPH – radical scavenger test (%) DPPH – test hvatača radikala	NO - radical scavenger test (%) NO – test hvatača radikala	FRAP (mg TE/g FW)
PEOD	4.24±0.20 ^{c}	17.57±0.44 ^{b}	6.02±0.38 ^{b}	43.99±4.17 ^{a}	56.15±2.46 ^{a}	32.72±1.80 ^{a}	93.90±4.07 ^{b}
PEOV	4.88±0.19 ^{b}	17.35±0.55 ^{b}	6.22±0.41 ^{b}	26.52±5.26 ^{c}	57.28±2.50 ^{a}	34.87±1.50 ^{a}	85.46±5.27 ^{b}
PLOD	5.51±0.12 ^{a}	33.81±1.39 ^{a}	9.38±0.44 ^{a}	35.61±6.21 ^{b}	41.69±3.03 ^{b}	27.95±2.34 ^{b}	203.50±20.35 ^{a}
PLOV	4.92±0.15 ^{d}	31.48±1.68 ^{a}	8.43±0.45 ^{a}	40.02±6.41 ^{a}	51.58±3.78 ^{a}	20.71±1.79 ^{c}	180.38±15.08 ^{a}

Key: pooled early oak vital – PEOV, pooled early oak damaged – PEOD, pooled late oak vital-PLOV and pooled late oak damaged – PLOD

Ključ: zbirni rani hrast vitalni – PEOV, zbirni rani hrast oštećeni – PEOD, zbirni kasni hrast vitalni – PLOV i zbirni kasni hrast oštećeni – PLOD

Table 3. Two-way Anova for phenological varieties, individuals and their interaction.

Tablica 3. Dvosmjerna Anova za fenološke sorte, jedinke i njihovu interakciju.

Parameter Parametar	Phenological variety Fenološki varijetet		Physiological groups Fiziološka grupacija		Phenological variety x Physiological groups Fenološki varijetet x Fiziološka grupacija	
	F	p	F	p	F	p
Proteins – *Proteini*	0.436	0.51 ^{NS}	3.97	0.05	5.751	0.001
Total phenolics content (TP) – *Totalni sadržaj fenola*	76.56	<0.001	1.97	0.16 ^{NS}	3.34	0.099^{NS}
Total flavonoids content (TF) – *Totalni sadržaj flavonoida*	27.77	<0.001	2.98	0.09^{NS}	0.754	0.648^{NS}
ABTS – radical scavenger test – *ABTS – test hvatača radikala*	3.03	0.09^{NS}	0.21	0.58 ^{NS}	5.910	0.001
DPPH – radical scavenger test – *DPPH – test hvatača radikala*	1.84	0.18 ^{NS}	5.23	0.02	4.822	0.033
NO – radical scavenger test – *NO – test hvatača radikala*	8.69	<0.001	0.22	0.64 ^{NS}	4.367	0.036
Ferric reducing antioxidant power (FRAP) essay – Esej antioksidativne moći redukcije gvožđa	28.85	<0.001	1.62	0.26 ^{NS}	14.751	<0.001

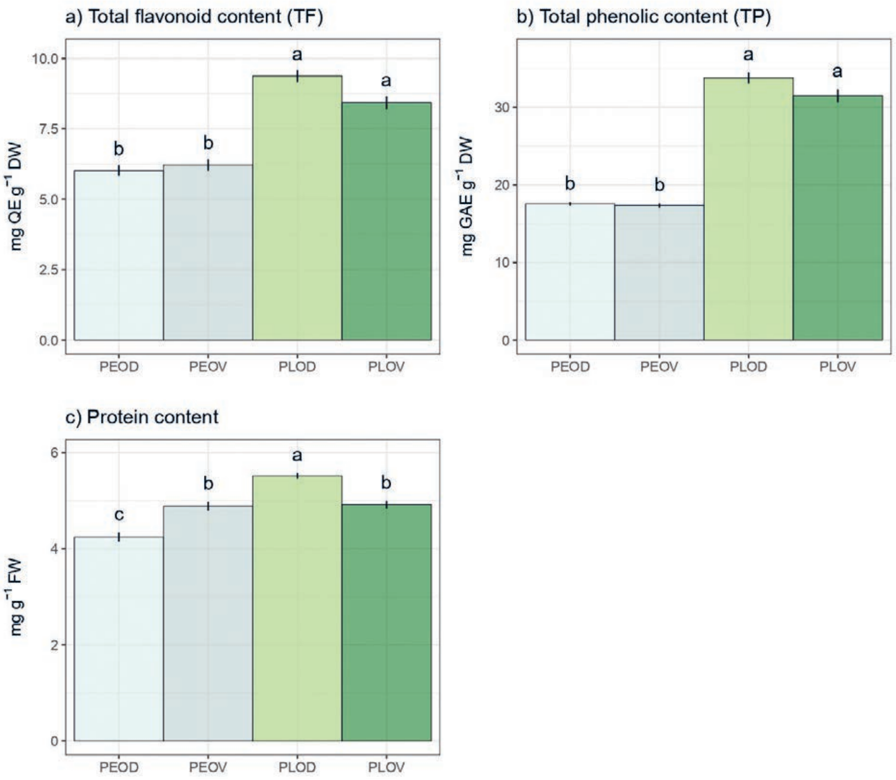

Figure 1. Total content of phenolics, protein and flavonoids within four physiological status groups of pedunculate oak
Graf 1. Ukupan sadržaj fenola, proteina i flavonoida unutar četiri fiziološke grupacije hrasta lužnjaka

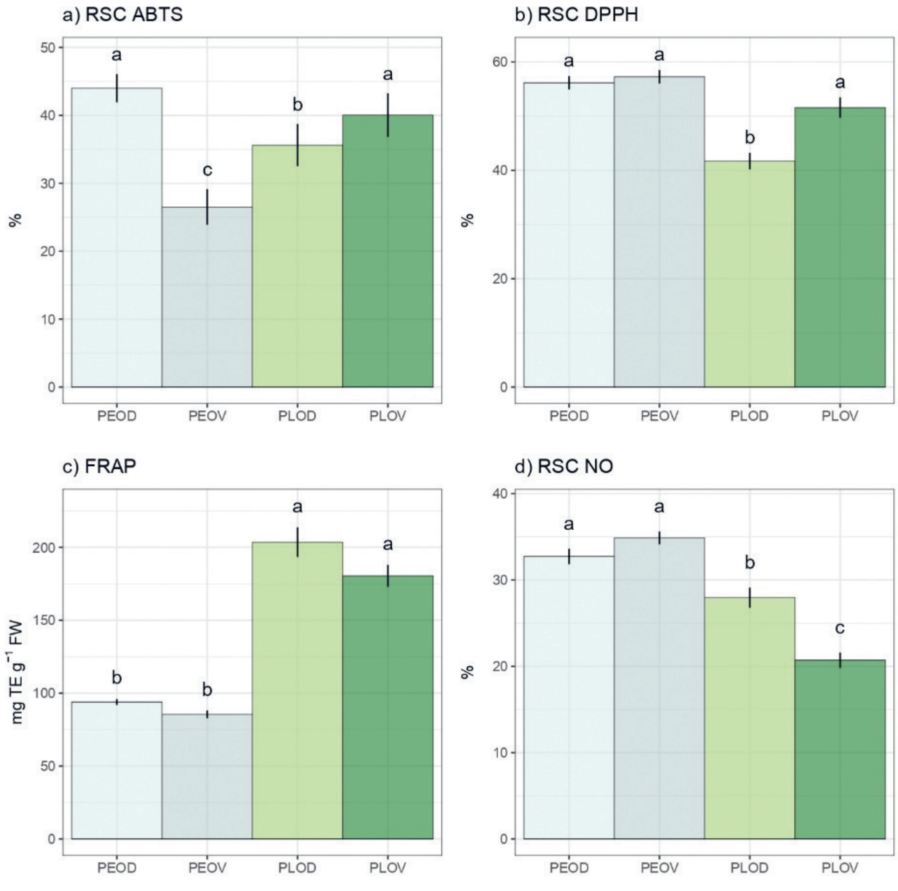

Figure 2. Radical scavenger capacity (ABTS, DPPH, FRAP and NO) within four physiological status groups of pedunculate oak
Graf 2. Kapacitet hvatača radikala (ABTS, DPPH, FRAP i NO) unutar četiri fiziološke grupacije hrasta lužnjaka

dical scavenger activity (Table 3). However, the results were affirmative for all other tested parameters, especially in combined effect of tested variables (based on the F test).

Principal Component Analysis (PCA) and Correlation Matrix – *Analiza Glavnih Komponenti (PCA) i Korelacijska Matrica*

The effect of phenological variety (early and late) and physiological groups (vital or damaged) on tested biochemical parameters were tested using PCA and Pearson`s correlation matrix for $p < 0.05$ (Figures 3A and 3B). Treatments were placed in different quadrants and samples distribution across both principal components (PC1 and PC2) was such that no grouping was observed. The PCA of tested parameters in oak samples (Figure 3A) explained that the first two principal components summarized 74.3% of the total variance in the parameters. FRAP and phenolic compounds showed a positive influence and DPPH-test showed negative influence on the PC1 coordinate, while ABTS-test showed a positive influence to PC2 coordinate computation. The late samples (PLOV and PLOD) highly corresponded with total phenolic compounds and TF parameters. A positive correlation was confirmed among proteins, total phenolic compounds and FRAP-test (Figure 3B).

DISCUSSION
RASPRAVA

The differences among early and late oak genotypes were more significant regarding the total phenolics and flavonoids contents, as well as ferric-reducing antioxidant power. This observation was also confirmed by PCA analysis where all three parameters were grouped together with a slight difference in the most dominant PC1 component. Phenolics found in plants are considered "quantitative" defenses due to their consistently high levels in plant tissue (Ruuhola et al., 2001). Genetically determined higher levels of phenolics in late oak genotypes represent constitutive protection against a wide range of abiotic and biotic challenges (Kebert et al., 2022b, 2022c). Two-way ANOVA in our case showed that physiological grouping had no influence on total phenolics content and subsequently on FRAP, ABTS- and NO- radical scavenger activity. Bojović et al. (2022) also examined several oxidative stress parameters in their study on pedunculate oak (FRAP, RSC against DPPH·, NO and ABTS radicals). Their results contributed to explaining differences of pedunculate oak mature trees in response to oxidative stress due to the intensity of the drought. Antioxidant capacities of examined individuals not only depend on plant, but also on the drought adaptation, which is closely related to the environmental factors in each microsite.

So far, in the forest research in Serbia, oxidative stress parameters were used in two cases: a) in the assessment of the phytoremediation potential of fast growing species from Salicaceae family (genera *Populus* and *Salix*) (Kebert et al., 2011; Trudić et al., 2012; Trudić et al., 2013; Pilipović et al., 2019) and b) in the efforts of the oxidative stress capacity assessment of certain broadleaved species (e.g. *Q. robur*, *F. sylvatica*, *C. betulus*, *P. avium*) while being exposed to osmotic and drought stress in controlled, semi controlled and open conditions (Štajner et al., 2013; Stojnić et al., 2016;

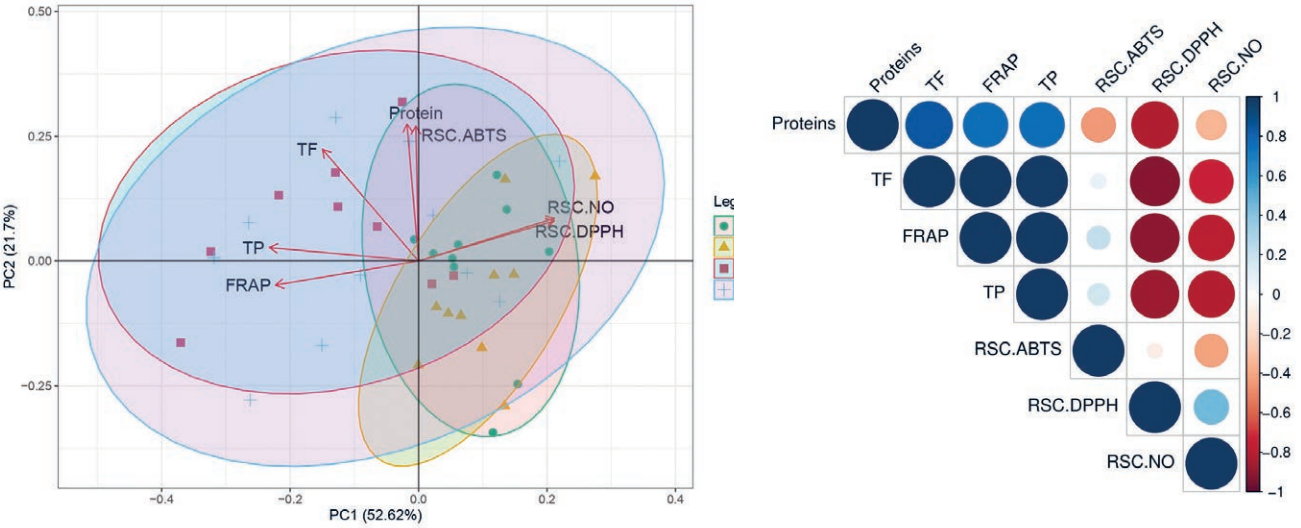

Figure 3. A) Principal component analysis two-dimensional scatter plot based on the first two principal components (Factor 1 and Factor 2) generated for tested biochemical parameters in four different physiological status groups of pedunculate oak. Different shapes represent individual trees in each tested group. B) Pearson's correlation matrix of analyzed biochemical parameters.

Graf 3. A) Dvodimenzionalni dijagram raspršenosti analize glavnih komponenti na temelju prve dvije glavne komponente (Faktor 1 i Faktor 2) generiran za testirane biokemijske parametre u četiri fiziološke grupacije hrasta lužnjaka. Različiti oblici predstavljaju pojedina stabla u svakoj ispitivanoj skupini. B) Pearsonova korelacijska matrica analiziranih biokemijskih parametara.

Stojnić et al., 2019; Stojnić et al., 2022; Kebert et al., 2022a; Vuksanović et al., 2022). The only studies which assessed oxidative stress and other parameters while being exposed to intense biotic stress (pest attacks) were conducted by Kebert et al. (2022a) (lipid peroxidation, total non-protein thiol compounds, Trolox Equivalent Antioxidant Capacity, FRAP, TP, TF and condensed tannins) and Pilipović et al. (2020) (combined stress experiment – drought and herbivore attack – where nitrate reductase and chlorophyll a content was measured, among others). These efforts were contributing to the selection activities, in order to identify individuals and provenances resilient to abiotic and biotic stress, in order to further develop breeding programmes of targeted species and contribute to climate smart forestry management practices in Serbia.

Depending on the species, other researchers have found contradictory results when studying the impact of drought and osmotic stress on polyphenol levels (Cheruiyot et al., 2007; Petridis et al., 2012; Griesser et al., 2015; Cherit-Hacid et al., 2015). For example, Štajner et al., (2011) investigated oxidative and drought stress tolerance in selected melliferous plant species (*Populus alba*, *Robinia pseudoacacia*, *Sophora japonica*, *Euodia hupehensis*, *Tilia sp*., *Fraxinus sp*.). During July, proline content and malondialdehyde quantity increased and soluble proteins decreased in all investigated species. The high and permanent antioxidant activity during the whole investigated period was observed in *P. alba*, but it was insufficient to protect its leaves from oxidative injury during the period of drought in July. The highest ability to accumulate proline and highest protein content under severe drought stress in July was observed in *Fraxinus sp*. Other investigated antioxidant parameters (total antioxidant and DPPH radical scavenger capacities) were high and accumulation of malondialdehyde was low which indicate high drought oxidative stress tolerance (Štajner et al., 2011). The study of Stojnić et al. (2016) showed that the content of free proline, FRAP units and the amount of malondialdehyde had increased values in *Q. robur* and *Carpinus betulus* trees subjected to soil water deficit. Stojnić et al. (2019) used oxidative stress parameters to identify traits that might be utilized to improve leaf-level intrinsic water use efficiency (WUEi), and therefore be used in breeding programmes to enhance drought adaptation of *Q. robur*. They analysed morphological, anatomical and oxidative stress parameters, where they screened total phenolics content, total flavonoids content, ferric reducing antioxidant power, total soluble protein content, radical scavenger capacity against ABTS•+, radical scavenger capacity against NO radical and radical scavenger capacity against DPPH free radical. However, the results of examined biochemical characteristics, presumably related to tolerance of oxidative stress, did not have considerable significance in conditions of moderate drought. Indeed, only FRAP was selected by stepwise regression analysis and, according to the results of path coefficient analysis, showed significant effect on the WUEi, both directly and indirectly through stomatal density and leaf dry mass per unit leaf area (Stojnić et al., 2019). Popović et al. (2017) investigated different poplar clones subjected to water stress and pointed out that phenolic compounds significantly or slightly decreased depending on the genotype. On the other hand, in the same paper, the increased biosynthesis of specific phenolic compounds, such as myricetin, chrysin, kaempferol and isoferulic acid etc., as well as increased activity of biosynthetic enzyme (PAL-phenylalanin ammonium lyase) under induced drought stress in poplar clones was reported. Recent research, however, has been in line with the results from our investigation that drought, among other stress factors, may cause the baseline level of phenolics to rise (Yoshida et al., 2015). However, the rise of phenolic compounds content and most of the antioxidant parameters was not significant in our groups of damaged oak. An exception was the ABTS radical scavenger test which discriminated damaged pooled early oak from vital, with a 66% increase of the scavengers of ABTS radical.

Trudić and Draškić et al. (2021b) described different candidate gene expression responses of two pedunculate oak phenological groups, characterized by different physiological status (vital vs damaged) and flushing period [early (var. *praecox*) vs late (var. *tardissima*)]. The most significant differences in relative gene expression levels are shown between the flushing period [*tardissima* (8 genes upregulated) vs *praecox* (3 genes upregulated)], more than a physiological status (vital vs damaged). This was also the case with our measurements of total phenol and protein content. Only three genes *wrky*53, *rd*22 and *sag*21 showed upregulated expression pattern in damaged physiological groups, indicating their possible role in the coping mechanisms of oak in stressed environment (Trudić and Draškić et al., 2021b).

Forest genetic monitoring, i.e. tracking of temporal changes in the genetic variation and structure of tree populations, is the only way to verify how well genetic diversity is maintained over time, and how this diversity is shaped by climate change and management practices. One of the first collaborative initiatives on defining comprehensive methodological framework of forest genetic monitoring on European continent was done by EUFORGEN group of experts (Aravanopoulos et al., 2015), although there was a history of similar initiatives in the past, mostly in other parts of the world (Namkoong et al., 1996; Boyle, 2000; Namkoong et al., 2002 (unpublished); Konnert et al., 2011). When detailing proper marker system and molecular data, Aravanopoulos et al. (2015) focused mainly on parameters accessible from DNA sequences, parameters of genetic diversity available from such data and quantitative morphometric data collected from various field trials and natural populations. There were no suggestions on biochemical or oxidative stress parameters as possible source of valuable data on genetic diversity and monitoring systems. Although detailed in plot design and morphometric analysis dataset needed for successful genetic monitoring of *Q.robur* (among others), in searched references on forest genetic monitoring there is no information on what exact molecular or biochemical/oxidative stress marker system might be used on pedunculate oak and others species as well (Fussi et al., 2016; Kavaliauskas et al., 2018; Bajc et al., 2020). This knowledge gap opens the space for expanding genetic monitoring methodology of forest tree species with biochemical parameters which will provide unique species-specific and landscape-specific information on the adaptation to climate change capacity of measured species population on site.

CONCLUSION
ZAKLJUČAK

Obtained results may help to explain differences in biochemical responses to abiotic stresses of pedunculate oak trees of different microsites and on the level of varieties within the single forest complex and provide us with valuable information about intra-species variability in drought resistance (early vs late). Therefore, this can be an important tool for the improvement of breeding strategies and artificial regeneration plans considering current circumstances and, more important, future climate scenarios. Given the results, the long-term measurement of oxidative stress parameters of oak trees on individual and population level is necessary in order to get clearer picture of what kind of changes happen on the molecular level. These measurements can also be useful in short term genetic monitoring of oak trees set up in semi-controlled and controlled conditions. This way it would be possible to get the insight into the exact genetic capacity of tree species regarding their abiotic stress resilience on certain microsites within the landscape. Considering all discussed results, it seems that oxidative stress parameters might be of species-specific significance and therefore, future genetic monitoring strategies should determine which of them are the most informative ones.

In addition, taking into consideration the significant amount of data presented in the discussion part, the next stage of forest genetic monitoring methodology development is to select oxidative stress parameters, physiological and biochemical data, which gave the most valuable information on diversity on individual and population level. Many of the mentioned molecular marker system, such as SSR, are selective neutral and have a disadvantage in framing a picture on a situational and short historical plasticity of the selected species in the landscape. This information is also very important for future breeding and conservation strategies and initiatives of valuable species like pedunculate oak, especially for small-scale forest management and planning.

ACKOWLEDGEMENT
ZAHVALA

This paper was carried out within the program of monitoring the impact of cross-border air pollution in forest ecosystems on the territory of AP Vojvodina in 2023 - financed by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia and supported by the PROMIS project entitled “MYCOCLIMART” No. 6066613 funded by the Science Fund of the Republic of Serbia. We would also like to thank Prof.Dr. Boris Popović, Faculty of Agriculture, University of Novi Sad, for comments significant for shaping of the discussion part of the paper. We would also like to thank Ms Gorana Kukobat, a court interpreter for the English language, for proofreading the translated version of this paper. FAO shall not be responsible for the content or any business activity related to this paper.

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SAŽETAK

Prema klimatskim scenarijima RCP-a, hrast lužnjak (*Q. robur* L.) označen je kao najteže pogođena vrsta drveća zbog stalnih, intenzivnih klimatskih promjena. Propadanje hrastovih šuma može uzrokovati velike ekonomske gubitke, budući da se hrastovi smatraju vrlo vrijednim primjercima drveća zbog duge povijesti iskorištavanja za proizvodnju drva i gradnju (Mikac i sur., 2018.). Uzimajući u obzir da uobičajeni biokemijski parametri mogu doprinijeti razumijevanju temeljnih obrambenih mehanizama biljaka protiv stresa od suše i stoga se koristiti kao pouzdani deskriptori prilagodljivosti u kontekstu klimatskih promjena, cilj ove studije bio je utvrditi postoji li neki od 7 predloženih testova praćenja oksidacijskog stresa (ukupni sadržaji bjelančevina, fenola, flavonoida i testovi hvatači slobodnih radikala – ABTS, NO, FRAP i DPPH) koji može poslužiti za dugoročni genetski monitoring dviju sorti hrasta lužnjaka (rani vs kasni). Fenološki varijeteti [rani (var. *praecox*) vs kasni (var. *tardissima*)] i fiziološka skupina (vitalne ili oštećene) bile su promatrane varijable u testiranih jedinki zbirnog radnog hrasta vitalnog (PEOV), zbirnog ranog hrasta oštećenog (PEOD), zbirnog kasnog vitalnog (PLOV) i zbirnog ranog oštećenog (PLOD). Svaka objedinjena (zbirna) skupina sadržavala je ukupno 10 ili 11 uzoraka, mjerenih u tri primjerka kako bi se postigla optimalna statistička značajnost za svaku skupinu (za više, pogledajte pododjeljak Statistika). Sakupljeno lišće odmah je označeno i stavljeno u aluminijski folij, konzervirano na suhom ledu i transportirano u Laboratorij za kemijske i biokemijske analize Instituta za nizinsko šumarstvo i okoliš (ILFE) na daljnju obradu. Dva grama hrastovog lišća homogenizirano je u ohlađenom mužaru s tučkom s 10 ml fosfatnog pufera (0,1 M KH_2PO_4, pH=7), centrifugirano 10 min na 11000 okretaja u minuti i supernatant je korišten za daljnji test praćenja oksidativnog stresa (Tablica 1), prema uputama autora. Sve analize određene su spektrofotometrijski u mikropločicama s čitačem Multiplate MultiScan GO (Thermo Scientific, Njemačka). Dobiveni rezultati biokemijskih parametara pokazali su da je lišće kasnog hrasta imalo veći sadržaj bjelančevina i fenolnih spojeva od ranog, bez obzira na fiziološku skupinu (vitalno ili oštećeno) (Tablica 2). Štoviše, radi boljeg prikaza rezultata iz Tablice 2. prikazali smo ih grafički (Graf 1. i 2.). Najveći udio ukupnih flavonoida imali su listovi PLOD i PLOV, a najmanji listovi PEOV i PEOD (Tablica 2). Što se tiče ispitivanja kapaciteta hvatanja radikala, svi testirani listovi imali su nizak kapacitet hvatanja. ABTS- i NO- testovi hvatači radikala pokazali su manje od 50% hvatanja za sve uzorke, dok je DPPH-test pokazao neznatno više od 50% hvatanja za PEOD, PEOV, PLOV listove (56,15%, 57,28% odnosno 51,58%) i 41,69 % za listove PLOD. Kasno lišće hrasta ima veću antioksidacijsku moć reduciranja željeza (203,5 odnosno 180,4 mg TE/g FW) nego rano (93,9 odnosno 85,5 mg TE/g FW), bez obzira na fiziološku skupinu (Tablica 2). Razlike između ranih i kasnih genotipova hrasta bile su značajnije u pogledu ukupnog sadržaja fenola i flavonoida, kao i antioksidativne moći reduciranja željeza (FRAP). Ovo zapažanje također je potvrđeno PCA analizom, gdje su sva tri parametra grupirana zajedno s malom razlikom u najdominantnijoj komponenti PC1 (Graf 3). Dvosmjerna ANOVA u našem slučaju pokazala je da fiziološko grupiranje nije imalo utjecaja na ukupni sadržaj fenola i naknadno na aktivnost hvatača radikala FRAP, ABTS i NO. Dobiveni rezultati mogu pomoći da se objasne razlike u biokemijskim odgovorima na abiotičke stresove stabala hrasta lužnjaka na različitim mikrolokalitetima i na razini varijeteta unutar jednog šumskog kompleksa te nam mogu pružiti vrijedne informacije o varijabilnosti unutar vrste u otpornosti na sušu (rano naspram kasno). Stoga ovo može biti važan alat za poboljšanje strategija uzgoja i planova umjetne regeneracije hrasta lužnjaka u sušnijim nizinskim regijama. S obzirom na rezultate, potrebno je dugoročno mjerenje parametara oksidacijskog stresa stabala hrasta na individualnoj i populacijskoj razini, kako bi se dobila jasnija slika o tome kakve se promjene događaju na molekularnoj razini. Ova mjerenja također mogu biti korisna u genetskom praćenju stabala hrasta postavljenih u polukontroliranim i kontroliranim uvjetima. Na taj način bi se mogao dobiti uvid u točan genetski kapacitet vrsta drveća u pogledu njihove otpornosti na abiotski stres na pojedinim mikrolokalitetima unutar krajolika.

KLJUČNE RIJEČI: antioksidacijsko, bioraznolikost, ekotip, hrast lužnjak, stres