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Tracing the responses of pedunculate oak (*Quercus robur* L.) trees to drought stress by analyzing the antioxidant system

Praćenje odgovora hrasta lužnjaka (*Quercus robur* L.) na sušu analizom antioksidativnog sistema

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Abstract: The influence of different groundwater table depths on pre-dawn water potential (PWP), soil moisture content and antioxidant activity (FRAP, RSC against DPPH·, NO and ABTS radicals) of adult pedunculate oak (*Quercus robur* L.) trees was assessed in three managed stands (localities 1-3) and one unmanaged (locality 4 - forest reserve). The study sites were located within single forest complex at different distances from the Sava riverbed. The measurements were performed during July 2015, when the first evidence of drought was evidenced. We hypothesised that the trees at locations farther from the river would be more drought-stressed due to lower groundwater depth and will show higher antioxidant capacity. Indeed, trees at localities 2 and 3 were more drought-stressed as compared to trees at locations 1 and 4, as indicated by the PWP results. Since antioxidant capacity can be directly correlated to drought tolerance, the present results indicate a higher antioxidant capacity in leaf extracts from locality 3, but also good oxidative adaptation in leaf extracts from locality 4, where the obtained values for almost all examined antioxidant tests were without statistically significant differences compared to those on locality 3. Obtained results may help to explain differences in biochemical responses to water deficit stress of pedunculate oak trees from different microsites within the single forest complex and provide us valuable information about intra-species drought resistance. Therefore, this can be an important tool for the improvement of breeding strategies and artificial regeneration plans for pedunculate oak in dry lowland regions.

Keywords: pedunculate oak, antioxidant activity, Sava riverbed, forest complex, drought.

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Sažetak: U ovom radu, procenjen je uticaj različitih dubina podzemnih voda na vodni potencijal lista (PWP), sadržaj vlage u zemljištu i antioksidativnu aktivnost (FRAP, inhibicija DPPH,·NO i ABTS radikala) odraslih stabala hrasta lužnjaka (*Quercus robur* L.) u tri gazdovne sastojine (lokaliteti 1-3) i u jednoj neuređenoj (lokalitet 4 – šumski rezervat). Lokacije istraživanja su se nalazile u okviru jedinstvenog šumskog kompleksa na različitim udaljenostima od korita Save. Merenja su obavljena tokom jula 2015. godine, kada su zabeleženi prvi dokazi o suši. Pretpostavili smo da će drveće na lokacijama udaljenim od reke biti pod većim sušnim stresom zbog manje dubine podzemne vode i da će pokazati veći antioksidantni kapacitet. Zaista, drveće na lokalitetima 2 i 3 bilo je pod većim stresom od suše u poređenju sa drvećem na lokacijama 1 i 4, kao što pokazuju rezultati PWP. Obzirom da antikosidativni kapacitet može biti direktno povezan sa tolerancijom na sušu, sadašnji rezultati ukazuju na veći antioksidativni kapacitet u ekstraktima listova sa lokaliteta 3 ali i na dobru oksidativnu adaptaciju listova sa lokaliteta 4, gde su dobijene vrednosti za skoro sve ispitivane antioksidativne testove bile su bez statistički značajnih razlika u odnosu na vrednosti dobijene na lokalitetu 3. Dobijeni rezultati mogu pomoći da se objasne razlike u biohmeijskim odgovorima na stres vodnog deficita kod stabala hrasta lužnjaka sa različitih mikrolokacija u okviru jedinstvenog šumskog kompleksa i daju nam dragocene informacije o intra-specijskoj otpornosti na sušu. Stoga, ovo može biti važna alatka za unapređivanje strategije oplemenjivanja i planova veštačke regeneracije hrasta lužnjaka u suvim ravničarskim regionima.

Ključne reči: hrast lužnjak, antioksidativna aktivnost, korito reke Save, šumski kompleks, suša.

INTRODUCTION

Complex of pedunculate oak forests in the Ravni Srem region are considered to one of the most valuable forests in Serbia. From the total area of pedunculate oak forest in our country (app. 65,000 ha), 29,081 ha is located in the lowland Ravni Srem region (Bobinac, 2008; Rađević et al., 2020). Unfortunately, the management of pedunculate oak forests in the last few decades is affected by a number of problems that are reflected in the dieback of individual and groups of trees throughout the entire area where this species occurs (Stojnić et al., 2014). Although a number of studies conducted in the Sava River basin (Medarević et al., 2009; Bauer et al., 2013; Stojanović et al., 2013; 2014a) 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).

From the perspective of climate change, Stojanović et al. (2014b), in their study which integrated climate change scenarios for the region of Serbia with the distribution of forest tree species, concluded that the most vulnerable tree species to the change of climate among the nine species with the highest abundance in Serbia is pedunculate oak. Likewise, in order to study the issue of oak floodplain forests response to water level, temperature and precipitation changes due to the altered climate conditions, Stojanović et al. (2015) found that the Sava River water level and the air temperature in April, May, June, July and August played a key role in the growth of pedunculate oak in the lowlands. According to Čater and Levanič (2015), growth of pedunculate oak in a floodplain forest depends mainly on the spatial and temporal distribution of precipitation during the growing season. Finally, Netsvetov et al. (2018) suggested that consistent patterns of relationships for floodplain Q. *robur* and climate variation are still mainly uncertain due to the various hydrological regimes over different sites coupled with the effect of regional climate trends.

From the ecophysiological point of view, pedunculate oak is an especially interesting species since its dependence on groundwater plays an uncertain role in its survival and stress response (Szatniewska et al., 2022). A number of studies demonstrated that the loss of soil water under drought in different tree species was followed by a decline of pre-dawn leaf water potential (PWP), since mentioned parameter provided estimates of both the water status of accessible soil water and the degree of plant water stress (Bréda et al., 1995). Thus, maintaining leaf water potential within an operational range is crucial to plant metabolism and survival, and the response of leaf water potential to soil water depletion plays a decisive role in overcoming drought conditions (Galmés et al., 2007).

Plants respond to the changes in the environment not only by altering their physiological processes but also adjust biochemically (Janiani et al., 2016). It is well known that drought conditions induce oxidative stress in plant cells. To protect themselves from this toxic condition, plants maintain a balance between reactive oxygen species (ROS) generation and consumption, which is strictly managed by the antioxidant defense system (Chutipaijit, 2016). Numerous researchers have proven that antioxidant capacity can be directly correlated to drought tolerance (Kebert et al., 2016; Rahimi et al., 2021). According to Kolarovič et al. (2009), drought-tolerant plants generally have a great antioxidant system capacity that increased various times over the unstressed plants compared with drought-sensitive plants in response to stress conditions. The present work focused on the complex assessment of the antioxidant properties of pedunculate oak leaf extracts using the different antioxidant assays, including DPPH$^{\cdot}$ and $^{\cdot}$NO radical scavenging capacities (RSC), FRAP and ABTS.

Although the impact of the Sava River water level on the growth and vitality of pedunculate oak trees in the floodplain forests in Srem is well-documented in literature (Stojanović et al., 2013, 2015), their biochemical responses to different soil water availability in relation to Sava riverbed distance, is scarce. Thus, the influence of different groundwater table depths on pre-dawn water potential (PWP), soil moisture content and antioxidant activity of adult pedunculate oak (Quercus roburL.) trees was assessed in three managed stands (localities 1-3) and one unmanaged (locality 4 – forest reserve), located at different distances from the riverbed, within single forest complex. The measurements were performed during the high growing season (20. July 2015), when the first evidence of drought was suspected due to extreme temperatures and stable riverbed conditions. The main objectives of our study were (1) to compare biochemical response (antioxidant and free-radical scavenging activities) of adult pedunculate oaks on four different locations in relation to the influence of the Sava river and groundwater table depths, and (2) to recognize and define tree response to drought. We hypothesized that the trees at locations farther from the river would be more drought-stressed due to lower groundwater depth and will show higher antioxidant capacity.

1. MATERIALS AND METHODS

1.1. Site description

Alluvial hygrophilic floodplain oak forests, situated in the Srem region, are spatially grouped into two approximately equal parts: the eastern ‘‘lower Srem’’ and the western ‘‘upper Srem’’ (both managed by the ‘‘Vojvodinašume’’ public forest enterprise; Stojanović et al., 2015). Four locations in the ‘‘lower Srem’’ were chosen for this research (Figure 1). The trail was conducted in three types of forests that make up about 65% representation of all forest types in Srem region (Galić et al., 2011). Typological characterisation of selected stands is described in Table 1.

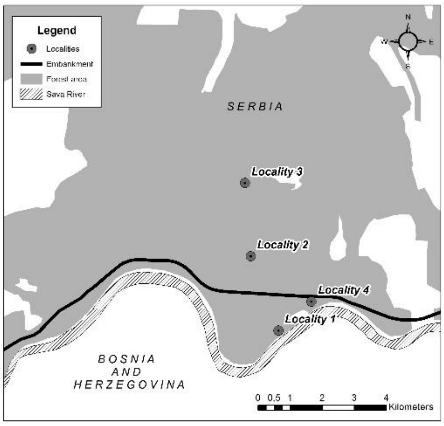

Figure 1 - Four different locations within single forest complex in Srem region

Table 1 - Types of forests where the trail was conducted

Locality	Forest type
1. Closest to the river	111 (VI3) – Forest of pedunculate oak, hornbeam and ash in floodplain (*CarpinoFraxino-Quercetumroborisinundatum*) on meadow blackaluvial brown soils (semigley) (https://soilgrids.org)
2. Intermediate	71 (IV2) – Forest of pedunculate oak and ash (*Fraxino-Quercetoroboristypicum*) on drier varieties of hydromorphic black soil (humosemigley) (https://soilgrids.org)
3. Farthest from the river	73 (IV4) – Forest of pedunculate oak and ash with common maple and tartar maple and rich shrub layer (*FraxinetoQuercetumroborisaceretosum*) on the driest varieties of hydromorphic soils and meadow black soils with the signs of lessivage (humosemigley to eugley with signs of lessivage) (https://soilgrids.org)
4. Forest reserve	111 (VI3) – Forest of pedunculate oak, hornbeam and ash in floodplain (*Carpino-Fraxino-Quercetumroborisinundatum*) on meadow black aluvial brown soils (semigley) (https://soilgrids.org)

1.2. Climatic data

Climate data (mean monthly temperatures and precipitation) was taken from the CARPATCLIM project database – CARPATCLIM Database ©European Commission – JRC, www.carpatclim-eu.org (Szalai et al., 2013). According to the World Climate Classification, studied area is described as temperate continental climate zone (Kottek et al., 2006). The coldest month was January, and the warmest was August and mean monthly temperatures range from ~0 to ~23°C. The monthly precipitation sum was highest in Jun (~44 mm), and lowest was in January (Figure 2).

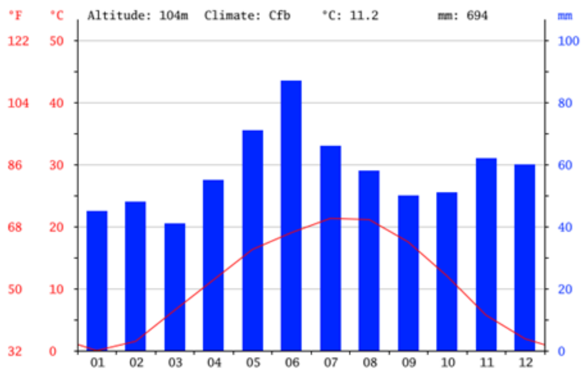

*Figure 2 - Climatic parameteres - monthly total precipitation and mean temperature in 2015 (*CARPATCLIM project database*)*

1.3. Pre-dawn water potential (PWP)

On each microsite at least three adults, dominating oak trees have been selected, where measurements of pre-dawn water potential (Ψpd) with Scholander-type pressure chamber (Plant Moisture Stress, Skye, UK) were performed. The measurements were carried out between 04:00 and 04:30 every day in first week of June to estimate soil water availability in the root zone. Sampling was made with a truck elevator platform, making sampling very transparent and representative from tree heights between 25-30 m above ground. From each tree at least three different branches from the upper crown were selected and from each branch at least four samples were measured to assure homogenous response. All values were corrected for the hydrostatic gradient (0.01 MPa/m) based on branch height above the ground.

1.4. Soil moisture content

Soil water content is expressed on a gravimetric basis. We analyzed soil moisture content (% mass) which determined on 10, 30 and 50 cm of soil depth. Gravimetric water content (θg) is the mass of water per mass of dry soil. It is measured by weighing a soil sample (m wet), drying the sample to remove the water, then weighing the dried soil (m dry) (Cooper, 2016).

1.5. Biochemical analyses, Extract preparation

About 0.2 g of air-dried powdered plant material was macerated with 70% ethanol (in water) in a ratio of 1:10 (w/v) for 24 h in the dark on a shaker and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was used for determination of radical scavenger capacity (RSC) against NO, DPPH and ABTS. For the determination of ferric reducing antioxidant power (FRAP test) and total soluble proteins of leaf samples were prepared by grinding 250 mg fresh plant material in 2 mL 50 mM K-phosphate buffer (pH 7.0) in a ground glass homogenizer. The homogenate was centrifuged at 15,000 g for 10 min at 4°C and the supernatant was separated.

DPPH radical scavenging assay

Ethanolic extracts of leaves were tested for their scavenging effect on the 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the method of Arnao (2000). Ten μL of plant extract were added to 290 μL of a 0.004% (w/v) solution of DPPH in 95% ethanol. The reaction mixture was shaken vigorously and the absorbance of the remaining DPPH was measured at 520 nm after 30 min. The radical scavenging activity was determined by comparing the absorbance with that of blanks (100%) containing only DPPH and solvent. All determinations were performed in triplicate. The DPPH radical scavenging capacity (RSC%) was calculated using the following equation:

$$RSC\% = ((A_{control} – A_{sample})/A_{control}) \times 100,$$

where $A_{control}$ is the absorbance of the reagent and A_{sample} is the absorbance in presence of the sample.

ABTS radical scavenging assay

Antioxidant activity of ethanolic extracts was estimated in terms of the $ABTS^{\bullet+}$ radical scavenging capacity following the procedure described by Miller and Rice-Evans (1997). Briefly, $ABTS^{\bullet+}$ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$solution (stable for 2 days) was diluted with 5 mM phosphate buffered saline (PBS, pH 7.4), to an absorbance at 730 nm of 0.70±0.02. After the addition of 10 μL of sample to 290 μL of diluted $ABTS^{\bullet+}$solution in microplate wells, the absorbance was measured after 30 min at 734 nm on microplate reader (Thermo Fisher Scientific, model Multiscan GO, USA). All samples were analyzed in triplicate and ethanol (95%) was used as a blank. Trolox with concentrations from 0 to 500 μM was used as a standard. The free-radical-scavenging activity was expressed as μmoles of Trolox equivalent (TE) per gram DW sample (μmol TE/g DW).

Assay of nitric oxide-scavenging activity

Nitric oxide (NO) radical inhibition was estimated by the GriessIllosvory diazotization reaction according to the method of Hensley et al. (2003). The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. NO scavengers compete with oxygen, leading to reduced production of nitrite ions. For the experiment, 60 µL 5 mM sodium nitroprusside, in 50 µL 0.1 M phosphate-buffered saline (PBS, pH 7.4) was mixed with 10 µL of ethanolic extracts and incubated at room temperature for 150 min. After the incubation period, 120 µL Griess reagent (1% sulfanilamide, 0.77 mM naphthyl ethylenediamine dihydrochloride, 2.5% H_3PO_4) was added. The absorbance of the chromophore formed was read at 546 nm. The NO radical scavenging capacity (RSC%) was calculated using the following equation:

$$RSC\% = ((A_{control}–A_{sample})/A_{control})\times100,$$

where $A_{control}$ is the absorbance of probes without the extract and A_{sample} is the absorbance of the reaction mixture with the sample.

Ferric reducing antioxidant power

The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe^{3+}\text{-}(TPTZ)_2]^{3-}$ to the intensively blue colored ferrous complex $(Fe^{2+}\text{-}(TPTZ)_2]^{2-}$ in acidic medium (Benzie and Strain, 1999). To perform the assay, 20 µl of leaf extract was added to 225 µl of FRAP reagent and 25 µl of water and shaken up for 20 seconds and the absorbance was recorded at 593 nm on microplate reader (Thermo Fisher Scientific, model Multiscan GO, USA). FRAP reagent was prepared freshly by mixing 20 mM $FeCl_3$x6 H_2O with 10 mM TPTZ (2,4,6-tripyridyl-S-triazine) and acetic buffer of pH 3.6 in ration of 1:1:10. All samples were analyzed in triplicate and ethanol was used as a blank. Trolox with concentrations from 0 to 500 µM was used as a standard. The free-radical-scavenging activity was expressed as mmoles of trolox equivalents (TE) per gram DW sample (mM TE g^{-1} DW) (Benzie and Strain, 1996).

1.6. Statistical analyses

Statistical differences were verified by the use of simple AVAR and LSD posterior analysis with standard 0.5 (*), 0.1(**) and 0.01(***) level of confidence. Data regarding RSC NO, RSC DPPH and RSC ABTS were subjected to the arcsine transformation, but actual percentages are given in the Figure 5.

2. RESULTS AND DISCUSSION

Values of PWP indicated smaller dispersion in both forest reserve and group that was closest to the river. Dispersion slightly increased with the distance from the water. Only group farthest from the riverbed (locality 3) indicated the beginning of the reversible water stress (-1.56 MPa), while other groups did not (Figure 3).

Among examined microsites, soil moisture content in all layers was the highest in locality 1 - site closest to the river (Figure 4), which was expected. However, although localities 1 (site closest to the river) and 4 (forest reserve) were characterized by the same forest type, the lowest values for soil moisture content in the deeper soil layers (30 and 50 cm) were observed in locality 4. Taking into account localities 2 and 3, SWC values in the deeper soil layers (30 and 50 cm) increased with the distance from the river (Figure 4).

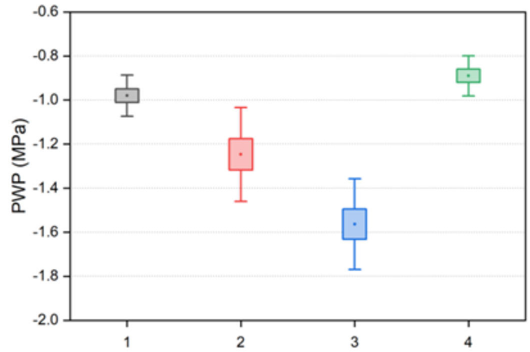

Figure 3 - Pre-dawn leaf water potential (PWP) in selected tree categories at the four examined sites. Bars are standard errors

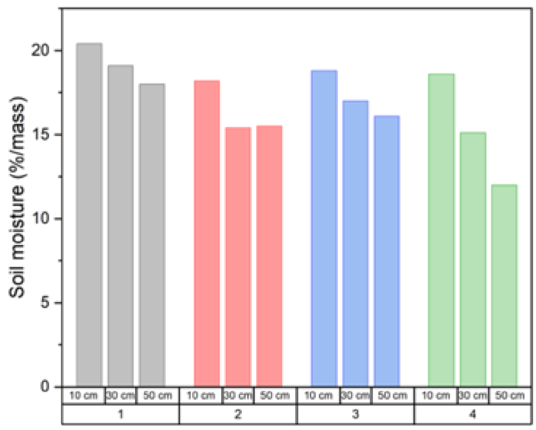

Figure 4 - Soil moisture content (% mass) at the four examined sites

The antioxidant activity of the leaves in the mature stands studied was analysed by the FRAP, DPPH, ·NO and ABTS tests (Figure 5). Response between microsites was evident, but did not followed the distance from the riverbed. Namely, in spite of comparable distance between microsite which was closest to the Sava River (locality 1) and forest reserve (locality 4), responses of trees in forest reserve indicated significantly higher values from the site closest to the water in almost all parameters of antioxidant status. In the intermediate group of trees (locality 2) obtained values for DPPH RSC and NO RSC were significantly lower than those from the forest reserve group (locality 4), while there were no statistically significant differences between mentioned groups regarding obtain results for ABTS and FRAP. On the other hand, due to the fact that only the group farthest from the riverbed (locality 3) indicated the beginning of the reversible water stress (-1.56 MPa, Figure 3), responses of trees in mentioned locality indicated (yet insignificantly) higher values compared to the forest reserve site (locality 4) regarding all examined antioxidant assays except ABTS.

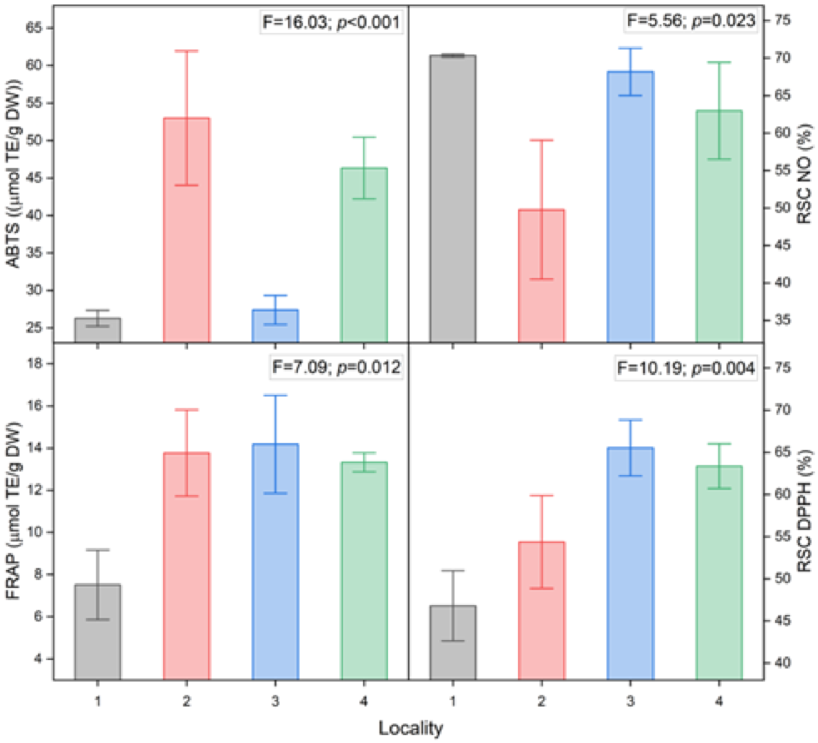

Figure 5 - Antioxidant assays of pedunculate oak leaf extracts at the four examined sites (from left to right): radical scavenger capacity against ABTS radicals, radical scavenger capacity towards NO; radical scavenger capacity against FRAP and DPPH radical scavenging capacity

A number of studies have shown that increased antioxidant concentrations are associated with increased stress (Haberer et al., 2008; Kebert et al., 2016). According to Rahimi et al. (2021), antioxidnt capacity can be directly correlated to drought tolerance since ROS scavenging system is an essential part of the protective mechanism against drought stress in plant cells. In the present study, development of drought stress, characterized by decreasing predawn water potential on microsite fatherest from the riverbed (locality 3) caused increase of total antioxidant activity estimated by FRAP, DPPH RSC and NO RSC test. Štajner et al. (2011) also detected that the highest induction of total antioxidant capacity determined by FRAP method, observed in *Fraxinus* sp., was in agreement with the accumulation of DPPH radical scavengers (DPPH RSC) in July, during drought period. According to Stojnić et al. (2016), who evidenced similar findings for non-irrigated pedunculate oak and hornbeam saplings, these results indicate that drought induces biosynthesis of chemical compounds with high antioxidant properties in order to cope with oxidative stress which is a direct consequence of drought. Furthermore, our results correspondents to the findings of Štajner et al. (2017) for beech population from Tara subjected to drought stress. They argued that this increase indicates good oxidative adaptation in examined population. Since antioxidant capacity can be directly correlated to drought tolerance, the values obtained in ABTS assay for leaf extracts from locality 3 indicated that it has low antioxidant capacity, which was not expected. According to Chaves et al. (2020) extracts showing poor antioxidant properties with one concrete method should not be discard as poor source of antioxidants without having been tested with other methods.

On the other hand, the obtained values for all examined antioxidant tests except ABTS in microsite forest reserve (locality 4) were without statistically significant differences compared to those microsite farthest from the riverbed (locality 3), indicating good oxidative adaptation of oak mature stands in forest reserve, also. Considering available data about soil properties at the forest reserve Stara Vratična (locality 4), it might be assumed that typical and haplic gleysol is characterized as the soil that is strongly affected by soil water (Kostić et al., 2021). Thus, although in forest reserve locality the lowest soil moisture content values in the deeper soil layers (30 and 50 cm) were observed, without the drop of pre-dawn water potential (PWP), the obtained values for all examined antioxidant tests (except ABTS) could be explained by the fact that before the water deficit has been established, roots have to sense the dry soil and transmit this information to shoot, which creates an integrated response of the plant allowing its survival until the water availability increases (Sanches and Silva, 2013).

As we mentioned above, in this study, examined oak tree in the microsite closest to the river (locality 1) was characterized by the highest moisture content in all examined soil layers, its PWP value did not indicate the beginning of the reversible water stress and obtained values for almost all parameters of antioxidant status were low except those for radical scavenger capacity towards NO radicals. However, current knowledge about free radical chemistry of nitric oxide ($^{\cdot}NO$) is scarce. Likewise, Popović et al. (2012) stated that radical-scavenging activity towards reactive oxygen species such as $^{\cdot}OH$, $^{\cdot}NO$, and $O_2^{\cdot -}$ radicals generelly proceeds by multiple different mechanisms and could be less selective, especially for $^{\cdot}OH$ because of its high reactivity.

Presented results can contribute to explain differences of pedunculate oak mature trees in response to oxidative stress due tothe intensity of the drought. Antioxidant capacities of examined pedunculate oak trees not only depend on plant but also on the drought adaptation which is closely related to the environmental factors in each microsite.

CONCLUSIONS

All the gained results confirmed that differences in the biochemical responses of adult pedunculata oak trees in relation to the distance of the Sava riverbed may explain adaptations of oaks on different sites within the single forest complex. Obtained results showed that response between microsites was evident, but regarding results obtained for antioxidant tests, did not followed the distance from the riverbed. The mature oak stand at locality 3 (farthest from the river) was undoubtedly affected by the reversible water stress, while for the trees from the other localities that was not the case.The present results indicate a higher antioxidant capacity in leaf extracts from locality 3, but also good oxidative adaptation in leaf extracts from locality 4. Obtain results may help to explain differences in biochemical responses to water deficit stress of pedunculate oak trees from different microsites within the single forest complex, which can be an important tool for the improvement of breeding strategies and reforestation programs for pedunculate oak in lowland regions.

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