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Full Length Research Paper

Screening of drought oxidative stress tolerance in Serbian melliferous plant species

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This study was designed to examine and compare antioxidant and free-radical scavenging activities of leaves of six different melliferous plant species (*Populus alba, Robinia pseudoacacia, Sophora japonica*, *Euodia hupehensis*, *Tilia* sp., *Fraxinus* sp.) from Serbia in order to evaluate their drought oxidative stress tolerance. Experiment was conducted during June, July and August. In this study, we reported the results concerning proline accumulation, soluble protein content, quantities of malonyldialdehyde, total antioxidant capacity determined by FRAP method and scavenger activity determined by DPPH method. According to our results, all melliferous plant species were subjected to drought oxidative stress during July when soil humidity decreased. During July, proline content and MDA quantity increased and soluble proteins decreased in all investigated species. High and permanent antioxidant activity during the whole investigated period was observed in *P. alba*, but 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 MDA was low which indicate high drought oxidative stress tolerance. Therefore, highest ability to adapt under severe drought stress and highest drought oxidative stress tolerance were observed in *Fraxinus* sp.

Key words: Melliferous trees, lipid peroxidation, DPPH, FRAP, proline accumulation.

INTRODUCTION

Forested ecosystems are being rapidly and directly transformed by the land uses of our expanding human populations and economies. Currently less-evident are the impacts of ongoing climate change on the world's forests. Enlarged emissions of greenhouse gases is now widely acknowledged by the scientific community as a major cause of recent increases in global mean temperature (about 0.5°C since 1970) and changes in the world's hydrological cycle including a widening of the Earth's tropical belt (Seidel et al., 2008).

Although a range of responses can and should be expected, recent cases of increased tree mortality and die-offs triggered by drought and/or high temperatures raise the possibility that amplified forest mortality has already occurred in some locations in response to global climate change. Examples of recent die-offs are particularly well documented for southern parts of Europe. Forest mortality due to dry and warm conditions in the 1990s and 2000s arcs across the Mediterranean regions, including increased death among many woody species in Spain (Peñuelas et al., 2001), increased mortality of oak, fir, spruce, beech, and pine species in France after the extreme heat wave and drought during the summer of 2003 (Landmann and Dreyer, 2006) and increases in mortality of *Pinus sylvestris* near the species' southern range limits in Switzerland and Italy. A severe drought in

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Abbreviations: DPPH, 1,1-Diphenyl-2-pycril-hydrasil radical; **RSC,** scavenging capacity; **FRAP,** ferric (Fe^{2+}) reducing antioxidant power; **MDA,** malondialdehyde; **ROS,** reactive oxygen species; $O^{2\bullet-}$, superoxide radical; **.OH,** hydroxyl radical; H_2O_2, hydrogen peroxide; **RO.,** alkoxyl radical.

2000 killed many *Abies cephalonica* in mainland Greece and *Pinus halapensis* sub. *Brutia* the most drought tolerant of the Mediterranean pines in Eastern Greece. Farther north, summer drought paired with biotic stressors has been linked to mortality of *Quercus robur* in Poland, *Picea abies* in Southeast Norway and with a severe die-off of *Picea obovata* in Northwest Russia (Allena et al., 2010).

Serbian trees are also exposed to a combination of environmental stress conditions, especially during the summer when low water availability is superimposed on high light and high temperatures at mid-day. Such combination of stresses, which are known as drought stress, may lead to an imbalance between antioxidant defenses and the amount of reactive oxygen species (ROS) resulting in oxidative stress (Smirnoff, 1993). Formation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and alkoxyl radical ($RO^{\cdot}$) by enhanced leakage of electrons to molecular oxygen are formed in high quantities during oxidative stress. Chloroplasts, mitochondria and peroxisomes are the major source of ROS in plant cells (Asada, 1999). Reactive oxygen species have long been proposed as signal molecules that regulate various processes such as growth, development, responses to biotic and abiotic environmental stimulation and programmed cell death (Mittler et al., 2004; Chung et al., 2008). However, at high concentrations, these ROS can be toxic by destroying normal metabolism through oxidative damage of lipids, proteins and nucleic acids (Fridovich, 1986; Štajner et al., 2007). Oxidative damage in the plant tissue is alleviated by an action of antioxidant mechanism and high proline accumulation at low water potential (Štajner et al., 1995; Xiong et al., 2001). There are many reports in the literature that underline the intimate relationship between enhanced or constitutive antioxidant and scavenging activities of plants and increased resistance to drought stress (Štajner et al.,1993, 1995; Türkan et al., 2005) Supporting this idea, enhanced antioxidant defense under drought stress was also reported in drought tolerant Mediterranean plants such as oak (*Q. robur*) (Schwanz and Polle, 2001), strawberry tree (*Arbutus unedo*) and olive tree (*Olea europeae*) (Sofo et al., 2004).

Therefore, the present study was designed to examine antioxidant (using FRAP method) and free-radical scavenging capacity (using DPPH method), lipid peroxidation intensity and proline accumulation in leaves of Serbian melliferous trees in order to examine their potential in enhancing protection from oxidative stress and drought. Obtained results should be used in selection of drought tolerant tree species.

MATERIALS AND METODS

Plant material

We studied 6 melliferum plant species-trees (*Populus alba, Robinia pseudoacacia, Sophora japonica*, *Euodia hupehensis*, *Tilia* sp., and *Fraxinus* sp.). Plant material was collected during June, July and August, from the greenwood nursery Kać. Samples were collected from three trees, from lower and medium region and the average sample was made. The age of each tree from experimental area was 11 years. The most common soils type at the experimental field was fluvisol (Galić et al., 2009). Fresh leaves were used for the experiment.

DPPH radical scavenging capacity (RSC) assay

One gram (1 g) of fresh herba was macerated with 10 cm^3 of absolute ethanol. After filtration, the ethanolic extract was used for the determination of 1, 1-diphenyl-2-pycril-hydrasil radical (DPPH) radical scavenging capacity (RSC). Reduction of DPPH radical was determined by measuring disappearance of DPPH at 515 nm. RSC is expressed in percentage compared to the control (Abe et al., 1998). The percentage inhibition of the DPPH radical (RSC) by the samples was calculated using the formula:

$$RSC = [(A_c - A_x)/A_c] \times 100\%$$

Where A_c is absorbance of the control and A_x is absorbance of the sample after 30 min of incubation.

Ferric (Fe^{2+}) reducing antioxidant power (FRAP) assay

Total antioxidant capacity was estimated according to the FRAP (Ferric Reducing Antioxidant Power) assay (Benzie and Strain, 1999). Total reducing power is expressed as FRAP units. FRAP unit is equal with 100 µmol/dm^3 Fe^{2+}. FRAP value was calculated using the formula:

$$FRAP\ value = \Delta A_{sample}/\Delta A_{standard}$$

Lipid peroxidation (LP) determination

Lipid peroxidation (LP) was determined by the thiobarbituric acid (TBA) method. Values were given as equivalent amounts of malonyldialdehyde (MDA). The calibration curve was prepared with malonyldialdehyde bis-diacetal (Placer et al., 1968).

Soluble protein determination

Soluble protein content was determined by the method of Bradford (1976).

Proline content determination

Proline accumulation was determined by the method as described by Paquin and Lechasseur (1979). Proline was determined after extraction with sulphosalicyclic acid, and reaction with ninhydrin. A standard curve of proline was used for calibration.

Statistical analysis

All determinations were performed in triplicate. Results were expressed as mean ± standard error. Statistical comparisons between samples were performed with Student´s t-test for independent observations. Differences were considered significant at $p<0.05$.

Table 1. Average month temperature and soil humidity during June, July and August.

Month	Average month temperature (ºC)	Soil humidity (%)
June	18.6	27.59
July	21.6	12.3
August	22.1	21.86

Table 2. Free proline accumulation in leaves of melliferous plants.

Plant species	Free proline [nmol/mg protein]		
	June	July	August
P. alba White Poplar	138.45 ± 0.35	140.61 ± 0.43	75.29 ± 0.31
R. pseudoacacia Black Locust	249.33 ± 0.71	425.58 ± 0.85	136.15 ± 0.42
S. japonica Japanese pagodatree	92.00 ± 0.22	230.88 ± 0.70	102.63 ± 0.40
E. hupehensis Bee bee tree	173.83 ± 0.49	177.97 ± 0.74	117.19 ± 0.30
Tilia sp. Lime	127.73 ± 1.39	173.06 ± 1.10	100.50 ± 0.99
Fraxinus sp. Black ash	110.28 ± 0.62	256.36 ± 0.63	140.07 ± 1.22

*Each value represents mean of triplicate studies. The highest value for each plant species was marked bold.

RESULTS AND DISCUSSION

Data concerning average month temperatures and soil humidity at the 70 cm deep are presented in Table 1. During July, the minimum soil humidity (12.3%) was observed. It was approximately twice smaller than in June (27.59%) and August (21.86%). Average temperature slightly increased from 18.6 to 21.86°C during the experimental period.

Our results were individually assessed for leaves of investigated *melliferum* plant species for their drought oxidative stress tolerance using proline accumulation, lipid peroxidation, soluble proteins and also by employing different tests for determination of free-radical antioxidant and scavenging capacities (using FRAP and DPPH methods).

Table 2 presents the results concerning free proline accumulation in leaves of investigated plants. The highest free proline quantity for all investigated *melliferum* plant species was detected during the drought conditions in July when free proline quantity ranged from 140.61 nmol/mg protein (in *P. alba*) - 425.58 nmol/mg protein (in *R. pseudoacacia*). Numerous studies have shown that the proline content in higher plants increases under different environmental stresses such as drought, high Salinity, high light and UV irradiation, heavy metals, oxidative stress and in response to biotic stress (Szabados and Savoure, 2010; Štajner et al., 1995). Proline accumulation in the leaves during water deficit was also reported in wheat (Pandey, 1982), in barley (Hanson et al., 1977) and in sorghum (Al-Karaki et al., 1996). Accumulation of proline under stress protects the cell by balancing the osmotic strength of cytosol with that of vacuole and external environment (Aspinall and Paleg, 1981). It also can interact with some enzymes and stabilize their structure and function. Turkan et al. (2005) observed higher proline accumulation in drought-tolerant than in drought sensitive species. Higher proline accum-ulation in drought tolerant species caused relatively higher water retaining capacity.

The changes in lipid peroxidation intensity expressed as nmol MDA/mg protein are presented in Table 3. The increase of lipid peroxidation intensity in all investigated melliferum plant species was observed in July. Malondialdehyde (MDA) quantity ranged from 24.45 nmol/mg protein (*S. japonica*) to 184.67 nmol/mg protein (*P. alba*). The maximum lipid peroxidation intensity and minimum proline accumulation were observed in *P. alba* (Table 2). In other investigated species, which exhibited higher proline quantities, lipid peroxidation was supperssed, suggesting that proline possesses antioxidant protective effect. Other authors also reported that high proline quantity in drought tolerant species designates high efficiency of antioxidant system (Turkan et al., 2005). Some studies suggested antioxidant feature to proline acting as a singlet oxygen quencher and H_2O_2 scavenger and also that it can reduce lipid peroxidation (Szabados and Savoure, 2010).

Soluble protein contents of investigated melliferum plant species are shown in Table 4. The smallest soluble protein contents in all plants except *Fraxinus* sp. were observed in July, under drought conditions. In July, soluble proteins ranged from 3.98 mg/g (for *Tilia* sp.) to 6.92 mg/g (for *Fraxinus* sp.). Other authors also reported that water loss may cause decrease in soluble protein level (Rosinger et al, 1984; Pandey et al, 2006).

Our results concerning total antioxidant capacity determined by FRAP method are given in Table 5. In July, during drought period, it ranged from 14.24 FRAP units in *Tilia* sp. to 65.61 FRAP units in leaves of *R. pseudoacacia*.

Table 3. Malondialdehyde (MDA) quantity in leaves of melliferous plants.

Plant species	Lipid peroxidation [nmol MDA/mg protein]		
	June	July	August
P. alba White Poplar	66.29 ± 0.44	184.672 ± 0.63	49.83 ± 0.66
R. pseudoacacia Black Locust	49.184 ± 1.00	80.21 ± 2.63	20.48 ± 0.47
S. japonica Japanese pagodatree	6.08 ± 0.20	24.45 ± 1.35	15.06 ± 0.82
E. hupehensis Bee bee tree	14.85 ± 0.36	38.95 ± 1.58	21.79 ± 0.10
Tilia sp. Lime	15.45 ± 0.07	38.24 ± 1.74	29.26 ± 0.7
Fraxinus sp. Black ash	13.40 ± 0.10	30.33 ± 0.64	16.83 ± 0.73

*Each value represents mean of triplicate studies. The highest value for each plant species was marked bold.

Table 4. Soluble protein content in leaves of melliferous plants.

Plant species	Proteins (mg/g)		
	June	July	August
P. alba White Poplar	9.37 ± 0.06	5.46± 0.05	10.78 ± 0.05
R. pseudoacacia Black Locust	8.26 ± 0.11	4.71 ± 0.04	8.81 ± 0.08
S. japonica Japanese pagodatree	20.02 ± 0.20	5.31 ± 0.06	7.77 ± 0.04
E. hupehensis Bee bee tree	11.50 ± 0.25	4.25 ± 0.12	7.16 ± 0.08
Tilia sp. Lime	5.84 ± 0.01	3.98 ± 0.06	6.99 ± 0.09
Fraxinus sp. Black ash	7.28 ± 0.101	6.92 ± 0.08	5.15 ± 0.14

*Each value represents mean of triplicate studies. The highest value for each plant species was marked bold.

Table 5. Total antioxidant capacity in leaves of melliferous plants.

Plant species	FRAP (FRAP units)		
	June	July	August
P. alba White Poplar	71.24 ± 0.29	59.72 ± 0.26	69.41 ± 0.40
R. pseudoacacia Black Locust	42.45 ± 0.18	65.60 ± 0.36	49.35 ± 0.22
S. japonica Japanese pagodatree	39.45 ± 0.11	34.66 ± 0.38	47.02 ± 0.25
E. hupehensis Bee bee tree	33.94 ± 0.37	32.33 ± 0.27	75.04 ± 0.19
Tilia sp. Lime	14.66 ± 0.25	14.24 ± 0.12	24.69 ± 0.14
Fraxinus sp. Black ash	27.68 ± 0.18	57.39 ± 0.29	12.93 ± 0.09

*Each value represents mean of triplicate studies. The highest value for each plant species was marked bold and the smallest one was marked bold italic.

R. pseudoacacia, P. alba and *Fraxinus* sp. exhibited the highest antioxidant capacities in July, where accumulation of antioxidants was stimulated by drought stress. Also, in July, the lowest antioxidant capacities were observed in *P. alba* (59.72 FRAP units), *S. japonica* (34.66 FRAP units) *E. hupehensis* (32.33 FRAP units) and *Tilia* sp. (14.24 FRAP units). In all the investigated periods, total antioxidant capacity was relatively high in *P. alba* which is in agreement with results presented in Table 6. 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) (Tables 5 and 6). Other authors also detected accumulation of secondary metabolites with antioxidant activity under abiotic and biotic stresses (Zhu et al., 2009; Munne-Bosch et al., 2001).

Table 6 shows the results of the DPPH radical–scavenging capacity (RSC). Our results indicated that leaves of investigated plant species exhibited different RSC. In July DPPH RSC ranged from 13.16% (*Tilia* sp.) to 93.33% (*P. alba*). RSC capacity was the highest in leaves of *P. alba* compared to other melliferum plant species during whole investigated period (86.49% in June, 93.33% in July and 89.03% in August). We observed the highest RSC in *P. alba* and *Fraxinus* sp. during the period of drought stress in July. In contrast to the above mentioned results, in *E. hupehensis* and *Tilia*

Table 6. DPPH radical scavenger capacity in leaves of melliferous plants.

Plant species	RSC DPPH (%)		
	June	July	August
P. alba White Poplar	86.49 ± 0.96	93.33 ± 0.37	89.03 ± 0.61
R. pseudoacacia Black Locust	79.82 ± 0.59	42.85 ± 0.54	25.26 ± 0.40
S. japonica Japanese pagodatree	46.66 ± 0.39	17.57 ± 0.67	16.22 ± 0.08
E. hupehensis Bee bee tree	34.78 ± 0.74	20.86 ± 0.60	57.45 ± 0.78
Tilia sp. Lime	18.71 ± 0.59	13.16 ± 0.31	17.89 ± 0.54
Fraxinus sp. Black ash	29.06 ± 0.34	70.47 ± 0.38	10.52 ± 1.15

*Each value represents mean of triplicate studies. The highest value for each plant species was marked bold and the smallest one was marked bold italic.

sp., the minimum DPPH RSC was observed in July, when average month temperatures and soil humidity were unfavorable. Relatively stable organic radical DPPH has been widely used to evaluate the antioxidant activity of various samples (Jung et al., 2008). Zhu et al. (2009) observed the increase of DPPH RSC in *Bupleurum* sp. under the drought stress. It could be assumed that, the accumulation of antioxidants would be necessary for scavenging reactive oxygen species and to protect lipid membrane from oxidative stress in plants subjected to drought stress (Zhu et al., 2009).

According to our results, all investigated melliferous plant species under drought stress showed similar biochemical changes such as proline accumulation, increase of lipid peroxidation intensity and decrease of soluble protein content. Protection against drought and oxidative stress could be induced by various physiological and biochemical changes like activation of antioxidant system and accumulation of some secondary biomolecules with antioxidant activity which depends on plant species. The highest ability to accumulate proline and highest protein content under severe drought stress in July showed *Fraxinus* sp. Other investigated antioxidant parameters (total antioxidant and DPPH radical scavenger capacities) were high and accumulation of MDA was low which indicated high drought oxidative stress tolerance of *Fraxinus* sp. High and permanent antioxidant activity during the whole investigated period was observed in *P. alba*. In this plant, it was insufficient to protect leaves from oxidative injury during the period of drought in July, when the highest lipid peroxidation intensity and also minimum proline quantity was observed. This approves the central role of proline in plant resistance to drought.

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