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Drought-induced changes in the antioxidant system and osmolyte content of poplar cuttings

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Abstract

Poplars are widely utilized in the intensive and biomass production, as well as in breeding and environment protection programmes. This experiment was performed to investigate the effect of drought stress on poplar clones (M-1, PE19/66 and B-229). Poplar clones were grown hydroponically under controlled conditions and exposed to drought stress by applying polyethylene glycol (PEG) 6000. The plant samples were collected and separated into roots and leaves. For estimation of antioxidant status, activities of different antioxidant enzymes were determined (superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), glutathione peroxidase (GPX), glutathione reductase (GR) and ascorbate peroxidase (APX)), as well as antiradical power (ARP) against hydroxyl (˙OH) radical using ESR spin-trapping. The water stress parameters like proline (PRO) content, activity of proline dehydrogenase (PDH) and glycine betaine (GB) content were determined. Drought stress had significant effects on PRO and GB contents, SOD, APX and CAT activities when compared to control. All investigated extracts were determined as good inhibitors for ˙OH radical reduction, especially clone M-1where there was an increase of ARP against ˙OH radical in drought condition what could help to prevent or meliorate oxidative damage. Results indicated that the M-1 clone had a greater accumulation of substances for osmotic adjustment and a more efficient enzymatic detoxification cycle for eliminating the negative effects caused by ROS under drought stress than clones B-229 and PE19/66. This study provides valuable information for understanding drought-responsive mechanisms in leaves and roots of poplar clones M-1, B-229 and PE19/66.

Keywords: antioxidant enzymes, climate change, drought, glycine betaine, proline

Introduction

It has been estimated that nearly 45% of all the world agricultural land is affected by drought, which constrains plant growth and leads to considerable reduction of plant yields (Ashraf and Foolad 2007). Rising levels of atmospheric CO_2 and temperature as well as extreme weather events are related to global warming and aggravate the adverse effects of drought on crop production and quality, tree establishment and growth (Mantovani et al. 2014, AbdElgawad et al. 2015).

Plants can survive under changed environmental conditions through a variety of mechanisms which allow plant to modulate water transport in response to drought. It has been established that drought stress impairs numerous physiological and metabolic processes in plants including changes in the secondary metabolites in plant tissues (Gill and Tuteja 2010, Popović et al. 2016). Drought stress induces oxidative stress increasing generation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and the hydroxyl radical (˙OH) (Mittler 2002). Increased level of ROS triggers a wide variety of biochemical changes in trees which includes damages of membrane structure, protein denaturation and chlorophyll degradation (Štajner et al. 2011). For plants, the maintenance of redox homeostasis in tissues is very important, so plants increase activities of antioxidant enzymes (e.g. superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), glutathione reductase (GR)) and content of non-enzymatic antioxidants

(e.g. glutathione, carotenoids, tocopherols) to maintain low levels of ROS and protect cells from oxidation damages (Gill and Tuteja 2010). Also, synthesis and accumulation of osmolytes (proline and glycine betaine) can provide an effective physiological and biochemical mechanisms to maintain turgor and contribute to drought tolerance (Khoyerdi et al. 2016).

Populus species are commonly used as models to study the biological processes and tolerance mechanisms of plants in response to drought, involving assessments of morphology, anatomy, physiology and molecular biology (Street et al. 2006, Yang et al. 2009, Yin et al. 2009, Cohen et al. 2010, Tosens et al. 2012, Xu et al. 2018). A number of studies have reported that there are differences in drought tolerance among species, genotypes as well as between the sexes of poplars (Peng et al. 2012, Barchet et al. 2014, Li et al. 2015, Viger et al. 2016). Determination of antioxidant activity can be very important since the changes in activities of antioxidant enzymes and other parameters of the antioxidant system can reflect the degree of drought stress tolerance and differences between tolerance of genotypes and plant species toward water stress. According to Laxa et al. (2019) the capacity of plant to increase concentration of antioxidants and activities of antioxidant enzymes, may be a suitable marker of drought tolerance. However, research on the relationship between water deficit stress and antioxidant response in tree species, especially in root samples is very scarce.

Therefore, the present study provides an investigation of the effects of water deficit stress on the antioxidant defence system of poplar leaves and roots. Two clones of eastern cottonwood (*Populus deltoides* Bartr.) and one *Populus* × *canadensis* Moench clone were used as model species to study biochemical response to drought. Selected clones are of high economic importance to Serbia, as they are frequently used in plantations. So far, these clones were used in conventional breeding programmes, which rely on morphological and phenological characterization (Orlović et al. 1997). To characterize plant performance under osmotic stress, young poplar trees in hydroponic culture were exposed to two levels of PEG 6000 (100 mOsm and 200 mOsm). To investigate the tolerance potential of the roots and leaves, proline and glycine betaine levels, as well as activities of antioxidant enzymes such as SOD, CAT, POD, ascorbate peroxidase (APX), glutathione peroxidase (GPX) and GR were determined. Furthermore, the drought effect on antiradical power (ARP) against $^{\cdot}OH$ radical was investigated.

Material and methods

Plant materials and growth conditions

Cuttings of *Populus deltoides* Bartr. (two clones: B-229, PE19/66) and the M-1 clone of *Populus* × *canadensis* Moench (a naturally occurring hybrid of *Populus deltoids* × *Populus nigra*) were obtained from the Institute of Lowland Forestry and Environment (Novi Sad). Sampled healthy cuttings were soaked in tap water for 24 h and after that rooted in plastic containers filled with 10 times diluted Hoagland's nutrient solution (Hoagland and Arnon 1950), which was inspected every week and aerated continuously. Cuttings could grow under controlled condition (16 h light, 281.7 µmol photons m^{-2} s^{-1} and 8 h dark cycle; temperature, 25°C ±2°C). After 4 weeks, drought stress was applied. To induce 100 mOsm and 200 mOsm drought stress, two different PEG 6000 concentrations were applied 100 g l^{-1} and 143 g l^{-1}, respectively. The experiment was arranged in a completely randomized design with three replicates (pots) per treatment. After six days, leaves and roots were collected, partially stored at –80°C and partially dried for further analysis performed on fresh and dry weight basis.

Estimation of glycine betaine (GB) content

The amount of GB was estimated according to the method of Grieve and Grattan (1983) by grinding dry leaves and roots samples in a mill and extracting them with distilled water for 24 h at 25°C. Then the samples were filtered, and filtrates were diluted to 1:1 with 1 M H_2SO_4. Aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold KI-I_2 reagent was added and the reactants were gently stirred with a vortex mixture. The tubes were stored at 4°C for 16 h and then centrifuged at 10,000 g for 15 min at 0°C (Boeco U-320R, Germany). The supernatant was carefully aspirated, and the per-iodide crystals were dissolved in 9 ml of 1,2-dichloroethane. After 2 h, the absorbance was measured at 365 nm with Thermo Scientific Evolution 220 UV-Visible Spectrophotometer, using GB as standard and expressed in µmol g^{-1} dry weight (DW).

Estimation of proline content

The PRO content was estimated by the method of Bates et al. (1973), using L-proline as a standard. The plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 3,500 g for 15 minutes. Supernatant was used for the estimation of PRO content. The reaction mixture consisted of 2 ml acid ninhydrin and 2 ml of glacial acetic acid, which was boiled at 100°C for 1 h. After termination of reaction in an ice bath, the reaction mixture was extracted with 4 ml of toluene and absorbance was read at 520 nm and expressed in µmol g^{-1} fresh weight (FW).

Estimation of proline dehydrogenase activity

The PDH activity was determined according to the method of Rena and Splittstoesser (1975). Plant samples (0.5 g) were extracted with 1.5 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.1 mM cysteine and 0.1 mM EDTA and centrifuged at 15,000 g for 10 min at 4°C. The supernatant was again centrifuged at 25,000 g for 20 min at 4°C. Obtained enzyme extract was incubated in the reaction buffer containing 100 mM Na_2CO_3 – $NaHCO_3$ (pH 10.3), 20 mM L-proline, 10 mM NAD^+ at 28°C, and

then PDH dependent NAD^+ reduction was monitored at 340 nm. Proline dehydrogenase activity was expressed in $U \cdot mg^{-1}$ protein, where one unit is defined as the amount of enzyme catalyzing the formation of 1 μmol of NADH per min^{-1}.

Estimation of glutathione (GSH) content

The content of GSH was determined with the Ellman reagent at 412 nm, according to the procedure of Sedlak and Lindsay (1968).

Estimation of ARP-OH by ESR-spin trapping

Fenton reaction was used to produce hydroxyl radicals by mixing 0.2 ml 112 mM 5,5-dimethyl-1-pyroline-N-oxide (DMPO), 0.2 ml 2 mM H_2O_2, 0.2 ml H_2O, 0.2 ml 0.3 mM $FeCl_2$ (blank) (Čanadanović-Brunet et al. 2009). The influence of poplar extract on the formation and stabilization of ·OH radicals was investigated by adding 0.006 ml phosphate buffer extracts in the Fenton reaction system. ESR spectra were recorded after 2.5 min, with the following spectrometer (Brucker 300 E, Karlsruhe, Germany) settings: field modulation: 100 kHz, modulation amplitude: 0.222 G, receiver gain: 1×10^4, time constant: 81.92 ms, conversion time: 327.68 ms, centre field: 3480.00 G, sweep width: 100.00 G, x-band frequency: 9.64 GHz, power: 20 mW, temperature: 23°C.

The inhibition ratio was determined by the following equation:

$$ARP\text{-}OH(\%) = 100 \cdot \frac{h_o - h_x}{h_o} ,$$

where:

h_o – the height of the second peak in the ESR spectrum of DMPO-OH spin adduct of the blank,

h_x –was the height of the second peak in the ESR spectrum of DMPO-OH spin adduct of the probe.

The data were the average of three measurements.

Extraction and estimation of antioxidant enzymes

Antioxidant enzymes were extracted from fresh plant material (0.3 g) by grinding in a mortar and pestle with 10 ml 0.1 M phosphate buffer (pH 7.0). After centrifugation at 12,000 g for 15 minutes at 4°C, aliquots of the supernatant were used for the estimation (for biochemical assays). An aliquot of the supernatant was used to determine its protein content by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

SOD activity was estimated by the method of Giannopolitis and Ries (1977). Reaction mixture contained 2.6 ml of 50 mM sodium-phosphate buffer (pH 7.8), 100 μl 13 mM methionine, 100 μl 75 μM NBT, 100 μl 0.1 mM EDTA and 50 μl 2 μM riboflavine and 50-10 μl enzyme extract (50-10 μl distilled water in case of control). The reaction mixture was incubated under two 15 W fluorescent lamps for 15 min and stopped by placing the tubes in dark for 15 minutes, after which the absorbance was recorded at 560 nm. A non-irradiated complete reaction mixture served as a blank. One unit of enzyme activity was taken as that amount of enzyme which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

Catalase activity (CAT) was determined by following the decomposition of H_2O_2 at 240 nm (Aebi 1984). The guaiacol peroxidase (POD) activity was measured using the method of Kato and Shimizu (1987). The activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} at 470 nm for oxidized tetraguiacol polymer. One unit of GPX activity was defined as the amount of the enzyme extract which consumes 1 μmol of H_2O_2 min^{-1} mg $protein^{-1}$.

Ascorbate peroxidase (APX) activity was measured by monitoring the decrease in absorbance due to the oxidation of ascorbic acid at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) in a reaction mixture containing 0.2 M Tris/HCl buffer (pH 7.0), 0.25 mM ascorbic acid, 0.5 mM H_2O_2 and the sample extract as described by Nakano and Asada (1981).

Glutathione reductase (GR) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 5 minutes (Halliwell and Foyer 1978). The 2.3 ml assay mixture contained 0.24 M TRIS buffer (pH 7.8), 0.6 mM GSSG and the leaf extract. The assays were initiated by the addition of NADPH at 25°C. GR activity was expressed as μmol NADPH oxidized min^{-1} ($U \cdot mg^{-1}$ protein). Glutathione peroxidase (GPX) activity was determinate by the method of Chiu et al. (1976) used cumene hydroperoxide and GSH as substrate.

To carry out statistical analysis, the advanced analytics software package STATISTICA 13 for Windows was used (Dell 2015). The comparisons were performed with the Duncan *t*-test for independent observations. The correlation matrices for results obtained for leaves and roots were calculated, giving the correlation coefficients between different variables, i.e. the analytical parameters tested. The absolute value of the number in a matrix indicates the strength of the interdependence, while the sign indicates a positive or negative interdependence between variables. Differences were considered significant at $r > 0.95$ ($P < 0.05$) (Table 2 and 3).

Results

Osmotic adjustment is considered an important mechanism of survival, which allows the maintenance of water uptake and cell turgor under water deficit. Glycine betaine is a cellular osmolyte that plays an important role in plants under different types of environmental stress (Sakamoto and Murata 2002). Our results revealed that exposure to PEG 6000 had an increasing effect on GB content in leaves and roots in all investigated genotypes (Figure 1), but the effects were more expressed in root samples than in leaf samples. The most prominent increase appeared under the 200 mOsm stress in comparison to the control of the following genotypes M-1 (13.2-fold) and PE 19/66 (8.3-fold). Since roots were exposed to PEG 6000, it could be presumed that the dramatic accumulation of glycine betaine in this plant tissue is a plant response to changed osmotic po-

tential and it contributes to osmotic adjustment necessary for continuing plant growth in osmotic stress conditions. These data agree with those of Khoyerdi et al. (2016) who detected higher GB accumulation in roots than in leaves of pistachio. It has been reported that GB enhances antioxidant potential of plants. Although direct ability of GB to neutralize ROS has not been confirmed, in some investigations, where researchers used transgenic plants with ability to accumulate GB, it was indicated that GB had an important role in maintaining activity of antioxidant enzymes, especially enzymes of the ascorbate-glutathione cycle (Chen and Murata 2011).

Among the plant osmolytes, proline (PRO) represents one of the most accumulated osmolytes, playing a vital role in protecting plants against various abiotic stresses (Verbruggen and Hermans 2008). Under normal water conditions, the PRO contents of the three different poplar clones were not identical; the M-1 leaves, and roots contained the highest PRO contents, 0.29 µmol/g FW and 0.23 µmol/g FW, respectively (Figure 2). The PRO contents of the leaves from the three different clones significantly increased after drought stress ($P < 0.05$). The highest increase was observed in leaves of clone PE19/66 under the 200 mOsm treatment (1.8-fold). Proline content in roots decreased with the drought treatment, but there was a statistically significant increase in roots of M-1 clone under the 100 mOsm treatment. A greater proline accumulation in leaves than in the roots was observed under unfavourable environmental condition. Similar results were found in *Populus cathayana* (Li et al. 2015).

The proline degradation is mainly mediated by the enzyme PDH. Proline accumulation may be a result of induction or activation of enzymes of proline biosynthesis (such as Δ^1-pyrroline-5-carboxylate synthetase) or decreased proline degradation or utilization in protein synthesis, as well as enhanced protein turnover (Delauney and Verma 1993). In this study, the treatment with PEG 6000 caused significant suppression of PDH activity in leaves of clone M-1, which is consistent with accumulation of proline (Figures 2A and 2B). In leaves of the other two clones, PDH activity stayed at control level or decreased. The decrease (in the M-1 clone), increase (in PE19/66 clone) and unchanged PDH activity (in B-229) in roots of *Populus* clones indicate different ways that plants regulate proline metabolism in response to drought stress.

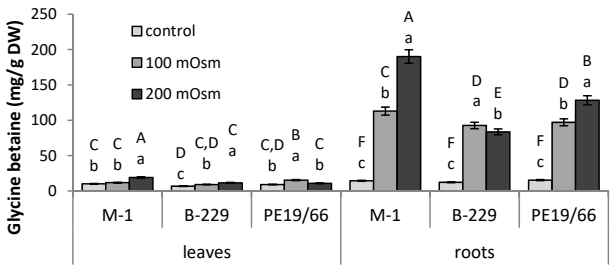

Figure 1. GB content in leaves and roots of poplar clones under different osmotic regimes

Each value is the mean ± SE (n = 3); the bars with different small letters among the same poplar clone are significantly different from each other ($P < 0.05$). Different capital letters on the bars indicate significant difference between clones ($P < 0.05$).

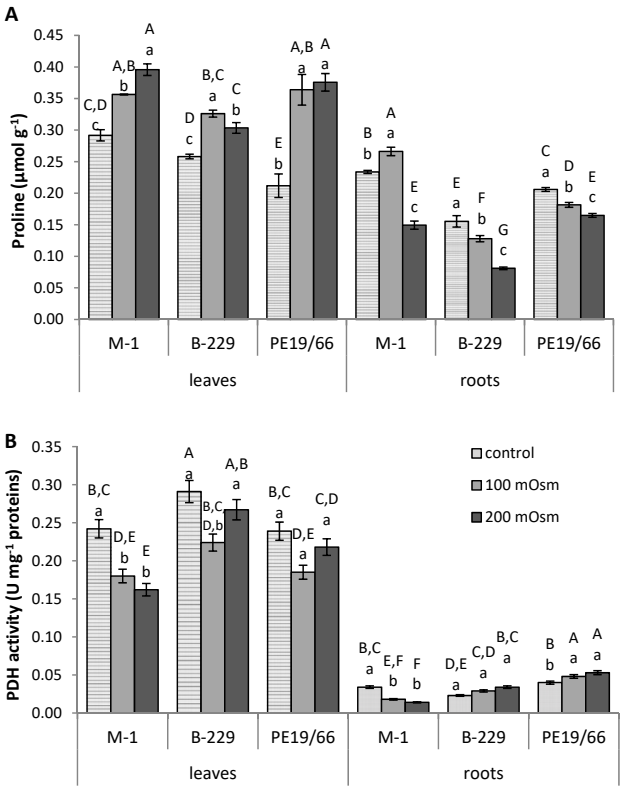

Figure 2. PRO content (A) and PDH activity (B) in leaves and roots of poplar clones under different osmotic regimes

Each value is the mean ± SE (n = 3); the bars with different small letters among the same poplar clones are significantly different from each other ($P < 0.05$). Different capital letters on the bars indicate significant difference between clones ($P < 0.05$).

Results concerning ARP-OH are summarized in Figure 3. In this study the spin-trapping reaction was used for detection of hydroxyl radicals in presents of poplar extracts to determine their antiradical power. Under normal water conditions, leaves of M-1 clone were highlighted by the highest ARP-OH. The exposure to 100 mOsm drought stress significantly increased ARP-OH in clones M-1 and PE19/66 (Figure 3A). Currently, there are other investigations of the antioxidant activity of poplar leaf extracts but there are no reports on antioxidant determination using the ESR technique on poplar extracts. It has been shown that ˙OH radicals may be responsible for oxidative damage during drought (Shen et al. 1997). The results obtained in this study showed that M-1 clone had high capacity to neutralize ˙OH radicals and this capacity rises in stress conditions. Different secondary molecules, including proanthocyanidins, might be influenced by severe drought stress. In our previous investigation (Popović et al. 2016), M-1 clone responded to osmotic stress by the significant increase of proanthocyanidine content, and due to their powerful an-

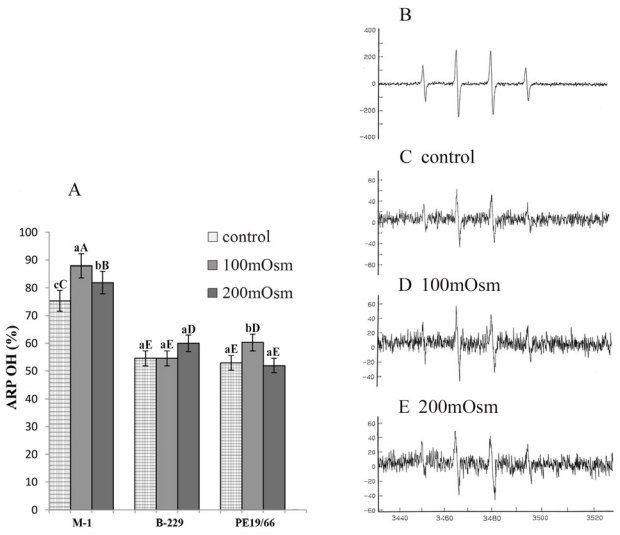

Figure 3. ARP-OH and ESR spectra of M-1 clone leaves as representatives of the most active clone against ˙OH radical

(A) ARP-OH under different stress (100 mOsm and 200 mOsm PEG 6000) in poplar clones M-1, B-229 and PE19/66; (B) blank signal (without plant extract); (C) signal of M-1, control; (D) signal of M-1, 100 mOsm PEG stress; (E) signal of M-1, 200 mOsm PEG stress.

tioxidant activity, increased ARP-OH could be used as a strategy to tolerate drought.

Table 1 summarizes results concerning GSH content and antioxidant enzyme activities of extracts. Under normal water conditions, the leaves of M-1 clone were highlighted by the greatest GSH content. The exposure to 100 mOsm drought stress significantly increased GSH content in leaves of clone B-229 (Table 1). Even though GSH quantity tended to increase in roots under drought treatment there were no significant changes, except under the 100 mOsm treatment of M-1 clone and under the 200 mOsm treatment of B-229 clone. Based on results obtained in this study, it could be suggested that moderate drought stress (100 mOsm) leads to increased amounts of GSH in poplar leaves and roots, and it can be assumed that this parameter contributes to oxidative stress tolerance caused by drought.

Regarding the enzymatic antioxidants, activity of SOD, CAT, POD, APX, GR, GPX were investigated and the results are listed in Table 1. For SOD activity, clone M-1 had the highest activities in control leaves and roots (Table 1). After drought stress, activity of SOD increased only in leaves of the B-229 clone, while it decreased in other tested samples of leaves and roots. In all investigated samples the activity of CAT stayed at control level, except the observed decrease in the leaves of M-1 clone under the 200 mOsm stress, the leaves of B-229 clone under the 100 mOsm stress and the roots of PE19/66 clone under the 100 mOsm stress (Table 1). The CAT activities of M-1 and PE19/66 reached their lowest values after the 200 mOsm drought stress treatment. Studying the CAT activity in different tree species, Alves et al. (2016) and Liu et al. (2010) have shown that this enzyme under drought treatment has a tendency to increase activity but under longer stress duration and higher stress intensity, decreased activity was observed.

In plants, POD has an antioxidant role in the biosynthesis of lignin by consuming hydrogen peroxide (Gill and Tuteja 2010). The POD is an important enzyme in the antioxidant system that prefers aromatic electron donors such as guaiacol and pyrogallol. Under normal water conditions in this study, the POD activity of the three different poplar clones were statistically different; M-1 leaves had the lowest POD activity, while PE19/66 clone had the highest POD activity and protein level, 4.20 and 14.49 U mg^{-1} respectively (Table 1). The statistically significant increase in POD activity was found only in the leaves of M-1 clone. In the other two clones, changes were statistically insignificant. In roots, POD activity was higher than in leaves, but decreased with drought treatment; only the roots of the PE19/66 clone stayed at control level.

The results concerning APX activity are shown in Table 1; the 100 mOsm treatment decreased APX activity in leaves of clones M-1 and B-229, but in leaves of PE19/66 a 9.9-fold enhancement was observed. The APX activity in leaves of all three clones was affected under 200 mOsm treatment; it increased by 1.5-fold in clone M-1, by 2.1 fold in clone B-229 and 16.4 fold in clone PE19/66. In roots, APX activity decreased with drought treatment; only in roots of the B-229 clone APX activity stayed at control level.

Gluthatione reductase is a functional part of the glutathione redox cycle. In this study, the GR activity in leaves of all three clones was not affected under drought treatment, except observed decrease under 200 mOsm treatment in PE19/66. In roots of investigated poplar clones GR activity have higher values than in leaves. Likewise, in leaves GR activity under drought treatment stayed at control level; only in PE19/66 under 100 mOsm stress GR activity decreased.

The GPX is an important enzyme in the antioxidant system that works with GSH and GR to prevent the formation of hydrogen peroxide. Increased antioxidant enzyme activities in leave samples could be used as a strategy to tolerate drought (Guerrier et al. 2000, Ahmed et al. 2013, Jovičić et al. 2017). In the control leaves samples GPX activity were observed: 0.011 (M-1), 0.018 (B-229) and 0.016 (PE19/66) U mg^{-1} proteins. GPX activity in leaves of M-1 and PE19/66 clones was increased under 200 mOsm treatment (by 1.9-fold and 1.75-fold). In root samples GPX activity under drought treatment decreased. In this study, activity of antioxidant enzymes seems to be well-balanced with glutathione content because the activities of all investigated enzymes except the CAT are higher in the roots than in leaves (Table 1), but the GSH content was much higher in leaves. Similar results were observed in chive plants where activities of POD and GPX were also higher in roots than in the leave tissue that indicated very active metabolism (Štajner et al. 2011).

The results of the correlation analysis are shown in Table 2 and Table 3. Significant correlations were observed between some antioxidant enzymes, while SOD in

Table 1. Effect of PEG 6000 on GSH content (μmol g^{-1} FW) and antioxidant enzyme activity (U mg^{-1} proteins) of leaves and roots in poplar clones M-1, B-229 and PE19/66

	Clone	PEG	GSH	SOD	CAT	POD	APX	GR	GPX
leaves	M-1	control	0.40$^{a\,A}$	32.24$^{a\,C}$	29.85$^{a\,B}$	4.20$^{b\,E}$	0.18$^{b\,D,E}$	0.02$^{a\,A,B}$	0.01$^{b\,E}$
		100	0.49$^{a\,A}$	28.11$^{b\,E}$	32.99$^{a\,A,B}$	5.72$^{a\,E}$	0.08$^{c\,E}$	0.01$^{a\,B,C}$	0.01$^{b\,D,E}$
		200	0.09$^{b\,C,D}$	29.25$^{ab\,D,E}$	14.06$^{b\,D}$	6.31$^{a\,D,E}$	0.27$^{a\,C,D}$	0.01$^{a\,B,C}$	0.02$^{a\,B,C}$
	B-229	control	0.25$^{b\,B}$	30.98$^{c\,C,D}$	36.53$^{a\,A}$	8.98$^{a\,C,D}$	0.42$^{b\,C}$	0.01$^{ab\,A,B}$	0.02$^{ab\,B,C,D}$
		100	0.45$^{a\,A}$	38.81$^{b\,B}$	30.36$^{b\,A,B}$	9.84$^{a\,C}$	0.34$^{b\,C,D}$	0.02$^{a\,A}$	0.01$^{b\,D,E}$
		200	0.19$^{b\,B,C}$	45.28$^{a\,A}$	31.69$^{ab\,A,B}$	11.63$^{a\,C}$	0.89$^{a\,A}$	0.01$^{b\,B,C}$	0.02$^{a\,B}$
	PE19/66	control	0.17$^{ab\,B,C,D}$	27.82$^{a\,E}$	20.95$^{a\,C}$	14.49$^{ab\,B}$	0.09$^{c\,E}$	0.02$^{a\,A}$	0.02$^{b\,B,C,D,E}$
		100	0.25$^{a\,B}$	22.13$^{b\,F}$	19.75$^{a\,C,D}$	11.50$^{b\,C}$	0.87$^{b\,B}$	0.02$^{a\,A,B}$	0.02$^{b\,C,D,E}$
		200	0.06$^{b\,D}$	19.17$^{c\,G}$	15.43$^{a\,C,D}$	17.46$^{a\,A}$	1.44$^{a\,A}$	0.01$^{b\,C}$	0.03$^{a\,A}$
roots	M-1	control	0.02$^{b\,C}$	118.5$^{a\,A}$	18.61$^{a\,A,B,C}$	40.90$^{a\,A}$	1.49$^{a\,B,C}$	0.10$^{a\,C}$	0.04$^{a\,B,C}$
		100	0.08$^{a\,B}$	65.8$^{c\,D}$	24.61$^{a\,A,B}$	29.50$^{b\,B}$	0.64$^{b\,D}$	0.12$^{a\,B,C}$	0.03$^{ab\,D}$
		200	0.02$^{b\,C}$	83.5$^{b\,B}$	25.68$^{a\,A}$	28.00$^{b\,B}$	0.77$^{b\,C,D}$	0.10$^{a\,C}$	0.03$^{b\,D}$
	B-229	control	0.06$^{b\,B,C}$	72.1$^{a\,C}$	17.00$^{a\,B,C}$	41.40$^{a\,A}$	0.91$^{a\,B,C,D}$	0.10$^{a\,C}$	0.05$^{a\,A,B}$
		100	0.11$^{b\,B}$	48.5$^{b\,F}$	16.06$^{a\,B,C}$	15.40$^{b\,C}$	1.14$^{a\,B,C,D}$	0.09$^{a\,C}$	0.03$^{b\,C,D}$
		200	0.18$^{a\,A}$	46.6$^{b\,F}$	17.99$^{a\,B,C}$	10.10$^{b\,C}$	0.79$^{a\,C,D}$	0.13$^{a\,B,C}$	0.03$^{b\,D}$
	PE19/66	control	0.06$^{a\,B,C}$	63.6$^{a\,D}$	25.58$^{a\,A}$	29.00$^{a\,B}$	2.36$^{a\,A}$	0.27$^{a\,A}$	0.06$^{a\,A}$
		100	0.08$^{a\,B}$	55.9$^{b\,E}$	13.99$^{b\,B,C}$	24.90$^{a\,B}$	1.60$^{ab\,B}$	0.08$^{b\,C}$	0.05$^{a\,A,B}$
		200	0.10$^{a\,B}$	52.2$^{b\,E,F}$	21.08$^{a\,A,B,C}$	26.40$^{a\,B}$	0.68$^{b\,D}$	0.20$^{ab\,A,B}$	0.03$^{b\,C,D}$

Values are means (n = 3); Means followed by different small letters within the same column, among the same poplar clone indicate significant differences ($P < 0.05$) according to Duncan's test. Means followed by different capital letters within the same column indicate significant differences ($P < 0.05$) according to Duncan's test. PEG, polyethylene glycol; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; POD, guaiacol peroxidase; APX, ascorbate peroxidase; GR, glutathione reductase; GPX, glutathione peroxidase.

leaves and GR in leaves and roots did not show any significant correlation. In leaves APX had a significant positive correlation with POD and GPX. The GSH concentration in leaves had a significant negative correlation with GPX, whereas proline content of leaves was significantly positive correlated with GB content and significantly negative correlated with PDH. In roots, significant positive correlations were observed between SOD and POD, and between APX and GPX, as well as significantly negative correlation between GSH and POD activity and GSH and SOD. In leaves, it was observed that activity of antioxidant enzyme APX correlates well with POD ($r = 0.691$), while SOD and CAT show high, but insignificant correlation with each other ($r = 0.637$). The significant correlation is also seen between CAT and GSH content (correlation coefficient, $r = 0.686$, Table 2).

It has been established by many authors (Ahmad et al. 2013, Ashraf and Foolad 2007) that GB has enhances antioxidant potential of plants, and in several studies a positive correlation of accumulation of GB and increased capacity of plants to neutralize ROS in stress conditions has been observed. In this study, all three clones accumulated GB in leaf samples followed by activation of different antioxidant enzymes: clone M-1 had a significant increase of POD, GPX and APX, while in other two clones APX activity increased. Moreover, Ahmad et al. (2013) noted that GB had an effect on induction of different genes, including mitigation of different stress conditions, regulation of signal transduction, metabolism and membrane transporters. In this study, significantly positive correlations in leaf samples were observed between GB and PRO, and between GB and ARP-OH ($r = 0.732$, 0.686, respectively), while in root samples that was not the case. In contrast, water stress in roots did not significantly change the activities of antioxidant enzymes. Among all investigated parameters in roots, however, only GSH and GB content increased significantly, especially in clones M-1 and B-229. This result indicates a crucial protective mechanism for ROS detoxifica-

Table 2. Coefficients of correlation between all investigated antioxidant parameters in leaves

	SOD	CAT	POD	GPX	GR	APX	GSH	PRO	PDH	GB
SOD	1.000									
CAT	0.637									
POD	–0.301	–0.413								
GPX	–0.229	–0.504	0.655							
GR	0.246	0.123	–0.140	–0.655						
APX	–0.256	–0.333	**0.691**	**0.767**	–0.651					
GSH	0.312	**0.686**	–0.602	**–0.856**	0.354	–0.542				
PRO	–0.306	–0.450	–0.117	0.316	–0.563	0.393	–0.101			
PDH	0.461	0.611	0.193	0.037	0.104	0.079	–0.001	**–0.759**		
GB	–0.223	–0.639	–0.204	0.199	–0.237	0.082	–0.339	**0.732**	**–0.810**	
ARP OH	0.082	–0.102	**0.737**	0.452	0.084	0.527	–0.438	–0.138	0.291	**0.686**

* Bolded values indicate significant correlation between parameters ($r > 0.95$, $P < 0.05$).

Table 3. Coefficients of correlation between all investigated antioxidant parameters in roots

	SOD	CAT	POD	GPX	GR	APX	GSH	PRO	PDH
SOD	1.000								
CAT	0.184								
POD	**0.752**	0.161							
GPX	0.130	–0.203	0.478						
GR	–0.231	0.550	–0.018	0.339					
APX	0.158	–0.003	0.163	**0.814**	0.455				
GSH	**–0.801**	–0.377	**–0.798**	–0.320	0.078	–0.287			
PRO	0.513	0.383	0.640	0.313	0.137	0.264	–0.622		
PDH	–0.302	–0.365	–0.168	0.398	0.415	0.381	0.321	–0.077	
GB	–0.271	0.289	–0.428	**0.717**	–0.239	–0.580	0.064	–0.209	–0.199

* Bolded values indicate significant correlation between parameters ($r > 0.95$, $P < 0.05$).

tion system in roots. It is well known that GSH participates in the regeneration of ascorbate from dehydroascorbate as well as in detoxification of different metabolites. On the other hand, GSH content increased in leaves only under the 100 mOsm stress condition, while in the 200 mOsm stress treatment GSH significantly decreased compared to control. The observed decreases of GSH can be explained by higher GPX activity, as well as the possible role of GSH in the detoxification processes.

Conclusions

The most important strategy for stress resistance and overcoming of water deficiency was the accumulation of proline and GB, because these parameters can be defined as the best indicators of poplar resistance to drought. Obtained results confirmed that water stress influences the antioxidant system, inducing the activity of certain antioxidants, as well as changes in proline metabolism and GB content. Differences in tolerance of the three tested clones to water stress were influenced by the PEG 6000 treatment. Clone B-229 was highlighted by activation of SOD and accumulation of GSH in leaves and roots under water stress condition caused by PEG 6000. Clone M-1 was highlighted by the activation of peroxidases under water stress conditions, as well as the greatest inhibitory effect on the formation of hydroxyl radicals. However, clone PE19/66 had the highest proline accumulation and the largest activation of APX. Results showed that poplar clones under water stress are stimulated to synthesize different enzymatic and nonenzymatic antioxidants. The results may be important for the selection of poplar clones resistant to water stress caused by PEG 6000. According to Laxa et al. (2019), the accumulations of antioxidant compounds and osmolytes are one of the key mechanisms for plants to handle with drought stress. Based on obtained data, clone M-1 could be recognized as promising clone, but further investigation under field conditions is necessary before any definite conclusion. This research could also lead to the identification of isoforms of antioxidant enzymes produced under conditions of water stress as well as some of these clones can be suitable for studies using genetic engineering to reduce drought sensitivity.

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