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A POSSIBLE ROLE OF SOME ANTIOXIDATIVE ENZYMES IN TOLERANCE OF *PAULOWNIA* TO NaCl

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Abstract

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The effect of salt stress on activities of glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPOX), total glutathione, ascorbate and H_2O_2 in three *Paulownia* lines (*P. elongata x fortunei x elongata* – T2, *P. elongata x elongata* – T4, *P. elongata* x kawakarnii – EK) grew as hydroponic culture at three levels of salinity, 50 mmol.l^{-1}, 100 mmol.l^{-1}, 200 mmol.l^{-1} sodium chloride (NaCl) solution was investigated. The magnitude of reduction of root, stem, leaf dry biomass and ratio total leaf area/ total dry biomass (LAR) were more pronounced in T4 than in T2 and EK after treatment. NaCl led to increase the leaf levels of H_2O_2, glutathione and ascorbate in T4 and T2, but not in EK. Salinity significantly inhibited the activities of CAT, GPOX and APX in T4. Increases in the enzyme activities by NaCl namely in T2 suggested that they might be playing a possible role in tolerance of this line to salinity. Our results suggested that T2 line was more tolerant to salt stress than EK and T4 lines. We were looking for suitable physiological and biochemical criterions in order to develop practicable strategy for selecting salt tolerant lines of *Paulownia*, which were produced by BioTree Ltd., Bulgaria.

Key words: hydroponic, protective enzymes, antioxidants, *Paulownia*, salinity

Introduction

High salinity affects the plants in different ways such as water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Shanker and Venkateswarlu, 2011). When the plants are under salt stress, its root and shoot length, fresh and dry weight and the leaf area are reduced, leaf chlorophyll content decreased and yield level fall (Zhu et al., 1997; Chinnusamy et al., 2007). The research to improve salt tolerance in plants is mainly focused on biochemical and physiological aspects and the genes responsible for salt tolerance in some species have been identified (Schleiff, 2008). The tissue culture and hydroponic methods could be useful in studying the salinity tolerance mechanisms in plants and their effects on plant production when are not evidently known.

Paulownia species are high-yielding trees that can be used for the production of energy, paper pulp and wooden building materials. These species is highly suitable to revalidate agricultural deserted areas, to reclaim mining areas, or to restore contaminated sites. Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production, and an effective way to capture genetic gains. Plants used in the current paper are propagated and rooted according technology registered by BioTree Ltd., Bulgaria. This laboratory is largest producer and supplier of genetically superior *Paulownia* tissue -cultures – *in vitro* seedlings. Our previous research with three *Paulownia* lines (T2, T4, EK), grown as hydroponic culture at different salinity levels showed that root and stem length, leaf number and total leaf area of T2 line are slightly reduced in comparison with these of T4 and EK lines, but the chlorophyll and carotenoid contents in the leaves of three clones remain relatively invariable (Miladinova et al., 2013).

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The present work aims at investigating the effect of NaCl on the dry biomass production and antioxidant defense in leaves of three *Paulownia* lines (*P. elongata x fortunei x elongata* – T2, *P. elongata x elongata* – T4, *P. elongata x* kawakarnii – EK), grown as hydroponic culture after transplantation the explants so as to provide fundamental base for vegetation restoration in salinized soils.

Materials and Methods

Plant material. Seeds and *in vivo* explants from the species *P. elongata* and their hybrids with *P. fortunei* and *P.* kawakarnii were used for developing of *in vitro* multiplication protocol as was described by Miladinova et al. (2013).

Hydroponic experiment. The experiments were set as four treatments including control, each treatment with 3 replications. The uniform seedlings were selected and transplanted to polyethylene vessels containing 1.2 l of 1/4 Hellriegel solution (Hellriegel, 1898) with an addition of A-Z microelements after Hoagland (pH 5.9) in growth chamber with a 16-h photoperiod (PAR 100 μmol m^{-2} s^{-1} on the upper leaf surface, 25/23±1ºC day/night temperature, relative humidity 60/70%). Each vessel contained two explants which represented one replication. The salt treatment was applied on the 48th day after transplanting of explants when the plants had adapted to the conditions of Hellriegel nutrient solution and 0 (control), 50, 100, and 200 mmol.l^{-1} NaCl was added. The solutions were aerated every day and were changed every 3 d to prevent depletion of nutrients and NaCl. Plants were harvested after 10 d of treatment. Toxicity symptoms (e.g. discoloration, pigmentation, yellowing and stunting) were assessed by eye through-out the experiment.

Measurement of plant growth. At the end of the experiment the plant samples were collected, washed with tap water and rinsed with distilled water before being separated into leaf, petiole, stem and root and fresh mass of each plant sample were measured gravimetrically. Dry mass of root, stem and leaf were determined after oven-drying (60ºC) for 2 days until constant weight was obtained. Leaf area was calculated using software program SigmaScan Pro 5.

Determination of enzymatic antioxidants. In order to prepare crude extracts for determination of enzymes glutathione reductase (GR), guaiacol peroxidase (GPOX) and catalase (CAT) the plant material were grinded with 4 ml of the extraction buffer (100 mM potassium phosphate buffer, pH 7.8; 5 mM EDTA; 2% PVP) that was added to 0.3 g of tissue powder. The extraction buffer for the determination of ascorbate peroxidase (APX) contained: 50 mM potassium phosphate buffer, pH 7.0; 1 mM ascorbate; 1 mM EDTA; 0.2% PVP and was added to 0.15 g of tissue powder. The suspensions were centrifuged (16 000g, 15 min, 4ºC). All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at 27ºC using Boeco S-22 UV/VIS spectrophotometer (Germany).

GPOX (EC 1.11.1.7) was estimated in reaction mixture of 100 mM potassium phosphate buffer (pH 7.0), 20 mM quaiacol, 200 μl extract, 1 mM H_2O_2. The oxidation of quaiacol was measured by following the increase in absorbance at 470 nm for 2 min (Polle et al., 1994).

CAT (EC 1.11.1.6) was estimated in reaction mixture of 100 mM potassium phosphate buffer (pH 7.0), 50 μ1 extract, 15 mM H_2O_2. The decomposition of H_2O_2 was determined by following the decline in absorbance at 240 nm for 3 min (Aebi, 1984).

GR (EC 1.8.1.7) was estimated in reaction mixture of 300 mM potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM GSSG, 200 μl extract, 0.15 mM NADPH. The oxidation of NADPH was determined by following the decline in absorbance at 340 nm for 3 min (Sherwin and Farrant, 1998).

APX (EC 1.11.1.11) was estimated in reaction mixture of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 200 μl extract, 0.5 mM NaAA. The rate of hydrogen peroxide-dependent oxidation of ascorbate was determined by monitoring the change in absorbance at 290 nm for 3 min (Nakano and Asada, 1981).

The protein content was determined after standard procedure of Lowry (1951).

Nonenzymatic antioxidant metabolites assays. For the low molecular antioxidant metabolites extraction, 0.2 g of FW of the fully developed leaves were ground into fine powder with liquid nitrogen, then 5 ml1 M $HClO_4$ were added. After 25 min centrifugation at 15 000 rpm at 4ºC, the supernatant was placed on ice and pH was adjusted to pH 7 (for glutathione) and pH 6 (for ascorbate) with 5 M K_2CO_3 (Doulis et al., 1997). The concentration of total (GSH+GSSG) glutathione was determined with an enzyme recycling assay (Griffith, 1980). The assay was based on sequential oxidation of glutathione by 5, 5`-dithiobis (2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of GR. The mixture in 1 ml contained 125 mM potassium phosphate buffer and 6.3 mM EDTA pH 6.5, 0.3 mM NADPH, 3 mM DTNB and 0.01 ml of the supernatant. The reaction was initiated by addition of 10 μl of GR (5 IU/ml) and the change in absorbance at $\lambda = 412$ nm was recorded. Standard curves were generated with reduced and oxidized glutathione. The results were expressed per 1 g FW.

Reduced form of ascorbic acid (Asc) was estimated as the decrease in absorbance for 1 min at $\lambda = 265$ nm, in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 5.6, 5 μl ascorbate oxidase and 0.02 ml supernatant. The reac-

tion was initiated with the addition of 0.02 ml of the supernatant and the decrease of the absorption of samples was recorded at λ = 265 nm. Standard curves were generated with Asc (Foyer et al., 1983). The results were expressed per 1 g FW.

For determination of H_2O_2 assay, 0.3 g FW of the fully developed leaves were homogenized in a mortar at 4°C with 3 ml 0.1% trichloroacetic acid and centrifuged for 20 min at 15 000 rpm. 0.5 ml of the supernatant were mixed with 0.5 ml phosphate buffer pH 7.4 and after the addition of 1 ml of 1 M KI, samples were incubated in the dark for 60 min and absorption was measured at λ = 390 nm. The content was calculated using a standard curve of H_2O_2 in the range of 1–100 nmol/ml of hydrogen peroxide (Jessup et al., 1984).

Statistical analysis

All values reported in this work were mean of at least three independent experiments. The mean values ±SD and exact number of experiments are given in the tables. The significance of differences between control and each treatment was analyzed by Fisher`s LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Results and Discussion

Effect of salt stress on seedlings growth. The seedlings growth is normally limited by increasing concentration of NaCl (Sreenivasulu et al., 2000). In our study, with increasing salinity levels, the root, stem and leaf dry mass in the three plants were reduced (Table 1). The leaf area ratios (LARs) are calculated in order to evaluate the capability of a plant in forming of photosynthetic surface. The control plants of T4 line possessed highest values of LAR. Highest LAR is established at 200 mmol/l NaCl for EK line despite of the lowest total dry mass. At the same concentration of NaCl increasing of LAR for T2 and decreasing of this parameter for T4 are observed as compared to the control (Table 1).

Effect of salt stress on seedlings protective enzyme activities. The increased accumulation of lipid peroxides and H_2O_2 are indicative of enhanced production of toxic oxygen. The results showed that salt stress produced more reactive oxygen species, resulting in more increased H_2O_2 and oxidative stress in T2 line at 50 mM l^{-1} NaCl. Highest values of H_2O_2 are observed in T4 at 100 and 200 mM l^{-1} NaCl (Figure 1A). The concentrations of total glutathione and reduced ascorbate are enhanced in the leaves of T4 and T2, but not in the leaves of EK line (Figure 1B and 1C).

The level of the antioxidant enzymes, such as superoxide dismutase (SOD), GPOX and CAT may determine the sensitivity of plants to lipid peroxidation (Kanazawa et al., 2000). In our study, activities of the antioxidant enzymes CAT, GPOX, APX and GR are enhanced by salt treatment of T2 plants (Figure 2A, 2B, 2C and 2D). With increasing salinity levels, CAT and APX activities declined in the leaves of T4 and EK plants (Figure 2A and 2C), but GR is enhanced (Figure 2D). Highest values of GPOX activity is observed in the leaves of EK line at 50 mM l^{-1} NaCl. The activities of investigated enzymes and the level of glutathione and ascorbate are highest in T2 line, which suggest that these

Table 1

Mean values ± SD (n = 5–6) of the root, stem and leaf dry mass, total leaf area/total dry mass (LAR) of three *Paulownia* lines (T4, T2, EK), grown as hydroponic culture in response to salt stress. Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P < 0.05)

Treatments	Root dry mass, g	Stem dry mass, g	Leaf dry mass, g	LAR, cm^2g^{-1}
T4				
Control	0.070±0.003c	0.059±0.018c	0.378±0.079b	800.05±21.68a
50 mM/l NaCl	0.158±0.013a	0.132±0.015b	0.367±0.041c	485.91±48.00d
100 mM/l NaCl	0.146±0.016b	0.153±0.017a	0.436±0.025a	512.57±15.85c
200 mM/l NaCl	0.071±0.004c	0.058±0.003c	0.193±0.084d	586.56±28.50b
T2				
Control	0.150±0.017a	0.180±0.017b	0.704±0.026a	482.11±32.00c
50 mM/l NaCl	0.127±0.016b	0.195±0.018a	0.503±0.021b	505.56±48,76b
100 mM/l NaCl	0.100±0.015c	0.121±0.015c	0.387±0.014c	559.41±46.36a
200 mM/l NaCl	0.075±0.006d	0.088±0.005d	0.295±0.018d	559.41±36.21a
EK				
Control	0.127±0.015b	0.110±0.008b	0.378±0.029b	556.85±24.04c
50 mM/l NaCl	0.135±0.012a	0.136±0.014a	0.411±0.043a	499.15±38.68d
100 mM/l NaCl	0.097±0.012c	0.081±0.009c	0.276±0.031c	603.71±91.75a
200 mM/l NaCl	0.079±0.006d	0.064±0.003d	0.198±0.010d	591.93±43.71b

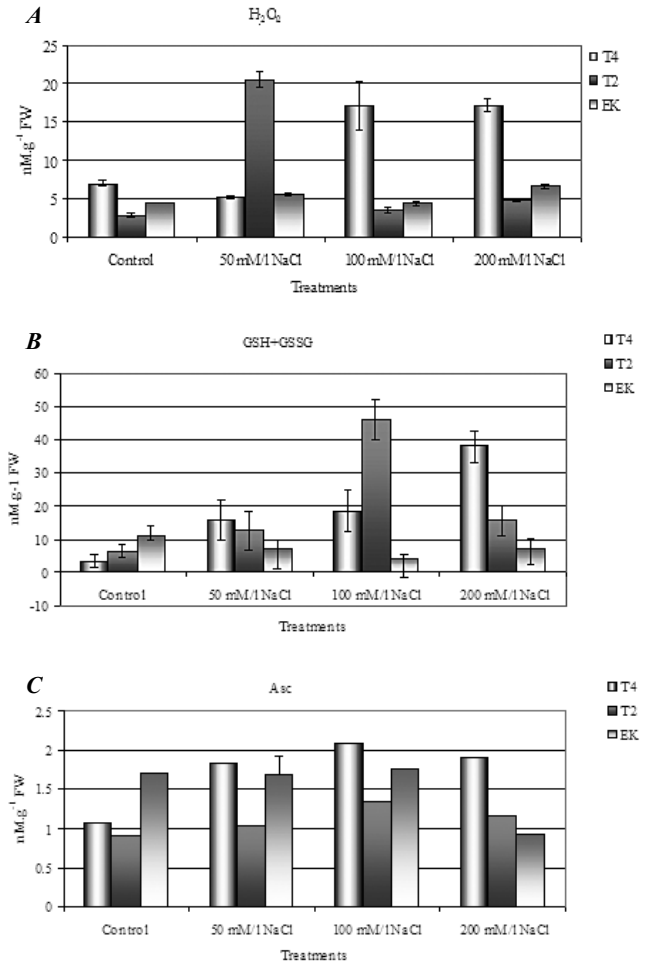

Fig. 1. Changes in H_2O_2 (*A*), total glutathione (*B*) and ascorbate (*C*) contents in the leaves of three *Paulownia* lines (*P. elongata x fortunei x elongata* – T2; *P. elongata x elongata* – T4; *P. elongata x* kawakarnii – EK), grown as hydroponic culture in response to salt stress

enzymes and low molecular antioxidants provide a better defense against salt stress – induced oxidative damage. The glutathione and ascorbate concentrations increased (Figure 1B and 1C), but activities of antioxidant enzymes changed in a different manner in the leaves of T4 line (Figure 2A, 2B, 2C and 2D). Lowest changes in the enzyme activities and antioxidant concentrations are observed in the leaves of EK line. Our previous investigations possessed that the root and stem length, as well as leaf number and total leaf area of T2 line are reduced insignificantly in comparison with these of T4 and EK lines during treatment with increasing concentrations of NaCl from 50 mM l^{-1} to 200 mM l^{-1} (Miladinova et al., 2013) The chlorophyll and carotenoid contents in the

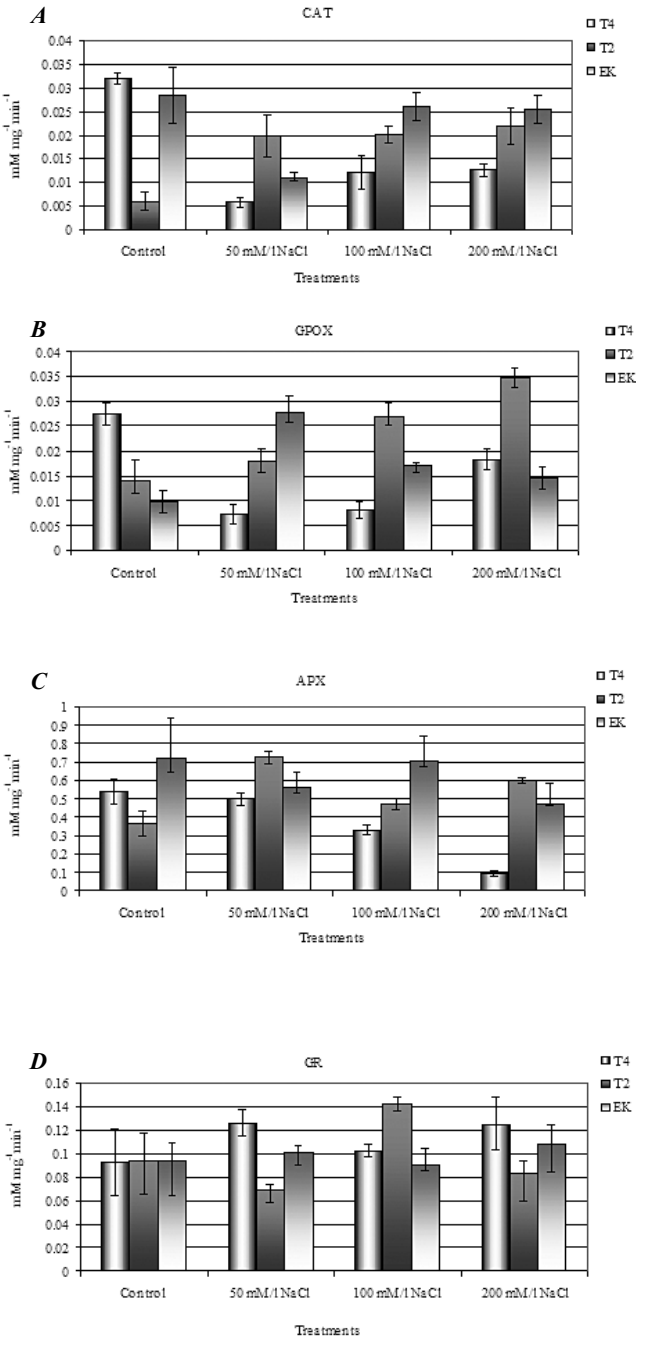

Fig. 2. Changes in catalase (*A*), quiacol peroxidase (*B*), ascorbate peroxidase (*C*) and glutathione reductase (*D*) activities in the leaves of three *Paulownia* lines (*P. elongata x fortunei x elongata* – T2; *P. elongata x elongata* – T4; *P. elongata x* kawakarnii – EK), grown as hydroponic culture in response to salt stress

leaves of this line remain relatively invariable and increased leaf area ratio (LAR) at 200 mM l^{-1} NaCl is observed in our experiments.

Conclusion

We examined the physiological and biochemical indicators in order to develop practicable strategie for selecting salt tolerant clones of *Paulownia* (*P. elongata x fortunei x elongata* – T2, *P. elongata x elongata* – T4, *P. elongata x* kawakarnii – EK), which were produced by BioTree Ltd., Bulgaria. *Paulownia elongata x fortunei x elongata* – T2 line was more tolerant to salt stress than EK and T4 lines. The results suggest a possibility to improve saline soil by utilizing *P. elongata x fortunei x elongata* – T2 line because it possesses better antioxidant defense and improves leaf area ratio (LAR) at highest NaCl concentrations.

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