Influence of salt stress on some physiological parameters of two Paulownia lines

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Abstract

Aim: We examined the physiological and biochemical indicators in order to develop practicable strategie for selecting salt tolerant lines of *Paulownia*, which were produced by BioTree Ltd., Bulgaria.

Material and Methods: An experiment was carried out to investigate the effect of salt stress on two *Paulownia* lines (*P. tomentosa x fortunei* – TF 01 and *P. elongata x fortunei* – EF 02) using three levels of salinity, 50 mmol/l, 100 mmol/l, 200 mmol/l NaCl solution. Dry mass of root, stem and leaves were measured gravimetrically, total leaf area – by using software program. Glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPOX), total glutathione, ascorbate and H_2O_2 were estimated spectrophotometrically.

Results: Under NaCl treatment, the root and stem dry mass of TF 01 line was reduced more in comparison with these of EF 02 line during. Activities of CAT, GPOX, APX and GR changed in a different manner in the leaves of TF 01 and EF 02 with increasing salinity levels.

Conclusions: *P. tomentosa* x *fortunei* – TF 01 was more tolerant to salt stress than *P. elongata* x *fortunei* – EF 02 line at the salinity conditions tested.

Keywords: *Paulownia*, salinity, growth, protective enzymes, antioxidants

Introduction

Natural or “primary salinity” is more widespread in arid and semi – arid regions of the world, while “secondary salinity” is due to land clearing or irrigation and continues to grow. Agricultural productivity in these regions is severely limited due to higher salinity in soil and water. The deleterious effects of salinity on plants are associated with: 1/ low osmotic potential of soil solution (water stress); 2/ imbalance of nutrients; 3/ specific ion effect (salt stress); or 4/ combination of these factors [8]. All these factors affected plant growth and development at physiological, biochemical and at the molecular level. The study of plant stress tolerance is suggested for understanding and transfer of tolerance traits to sensitive crop plants in future. Increased salt tolerance of perennial species used for fodder or fuel production will be a key component in reducing the spread of “secondary salinity”, while increased salt tolerance of crops will directly improve production in soils with “primary salinity” [10]. During the course of plant growth, the form and functions of various organs undergo significant changed, and the ability of the plants to react to salinity stress depends on those genes that are expressed at the stage of development during which the stress is imposed. The mechanism of salinity tolerance becomes more complicated when the response of a plant also varies with the concentration of saline medium and the environmental conditions in which the plant is grown. The plant cell and tissue culture methods could be useful in studying the salinity tolerance mechanisms in plants and their effects on crop production when are not evidently known.

Paulownia is native from China. *Paulownia tomentosa* has been introduced into USA and Europe as an ornamental plant and is still widely used for this purpose. Trees introduced in Bulgaria reach 12 m average height and 13.4 cm average diameter during 7 years. Over the last two decades *Paulownia* species has been extensively studied due to its ability to uptake nitrates and land contaminants, namely heavy metals. This high-yielding tree can be used for the production of energy, paper pulp and wooden building materials. The genetically tissue–cultured *Paulownia* seedlings produced by The World Paulownia Institute (WPI) allow production of biofuels after introducing of cultivars without detrimental impacts on food supply or the environment. Research on *in vitro* propagation of *P. elongata* and *P. fortunei* has been reported [2]. Application of this technology for micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and it is effective way to maintain the genetic gain [12].

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, which are preferred from the farmers due to its fast development and an uniform and regular

growth. Two lines (*P. tomentosa x fortunei* – TF 01 and *P. elongata x fortunei* – EF 02) are selected and patented with the purpose of obtaining two types of plants: *(i)* lower and branchy individuals and *(ii)* trees higher individuals, less branchy for the purpose of wood material formation. There is no information about salt tolerance of these lines and possibilities to use as phytoremediators of saline soils.

In this research, the effect of NaCl on the growth and antioxidant defense in leaves of two *Paulownia* lines (*P. tomentosa x fortunei* – TF 01, *P. elongata x fortunei* – EF 02), grown as hydroponic culture after transplantation the explants were compared 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 of *P. tomentosa* and *P. elongata* and their hybrids with *P. fortunei* were used for developing of *in vitro* multiplication protocol. For induction of shoots, explants were cultured on Murashige and Skoog (1962) nutrient medium included 2.5% (w/v) sucrose, 0.8% (w/v) agar and vitamins. For shoots multiplication MS medium supplemented with 4.439 μM 6-benzylaminopurine (BAP) and 0.537 μM indolilacetic acid (IAA) was used. After multiplication the shoots were transferred to rooting medium based on half strength basal salts MS medium, 2 % sucrose, 6 % agar and vitamins supplemented with 4.92 μM indole-3-butyric acid (IBA) and 1.075 μM IAA. The pH of all media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH before autoclaving. All cultures were incubated under controlled conditions – 16 h photoperiod, light intensity of 35 μmol m^{-2} s^{-1} and 24/18±1ºC day/night temperature. After three weeks of rooting, the shoots were rinsed with 1.5 ml/l Proplant solution.

Hydroponic experiment. The experiments were set as four treatments including control, each treatment with 5 replications. The uniform explants were selected and transplanted to polyethylene vessels containing 1.2 l of 1/4 Hellriegel solution (1989) 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±1 ºC day/night temperature, relative humidity 60/70%). Each vessel contained two plants which represented one replication. After 21 days of cultivation the plants were transferred to 1/2 Hellriegel solution with the addition of A-Z microelements (pH 5.9). The salt treatment was applied on the 48th day after transplanting of explants when the plants had adapted to the conditions of 1/2 Hellriegel nutrient solution and 0 (control), 50, 100, and 200 mM/l 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 (60°C) 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, 4 ^{o}C). All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at 27°C using Boeco S-22 UV/VIS spectrophotometer (Germany).

GPO (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 [13].

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 [1].

GR (EC 1.6.4.2) was estimated in reaction mixture of 300 mM potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM GSSH, 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 [14].

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 [11].

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 4º C, 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. The potassium perchlorate was removed by further centrifugation and the clear supernatants were used for the assays [3]. The concentration of total (GSH+GSSG) glutathione was determined with an enzyme recycling assay [5]. 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 λ = 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 λ=265 nm, in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 5.6, 5µl ascorbate oxidase and 0.02 ml supernatant. The reaction 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 [4]. 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 [6].

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

Seedlings growth is normally limited by increasing concentration of NaCl. In our study, the root and stem length of *Paulownia tomentosa x fortunei* line TF 01 is reduced more than that of *Paulownia elongata x fortunei* line EF 02. The leaf number of EF 02 rose, but total leaf area declined sharply with increasing concentration of NaCl, while these of TF 01 are not changed significantly [9].With increasing salinity levels, the root, stem and leaf dry mass of *P. tomentosa* x *fortunei* declined more than that of *P. elongata* x *fortunei* as compared to the control (Table 1). The leaf area ratios (LARs) are calculated in order to evaluate the capability of a plant in forming of photosynthetic surface. Highest value is established at 200 mmol/l NaCl for TF 01 line despite of the lowest total dry weight. At the same concentration of NaCl decreasing of LAR for EF 02 is observed as compared to the control (Table 1).

Table 1. Mean values ± SD (n = 5-6) of root, stem, leaf dry mass and total leaf area/total dry mass ratio (LAR) of *Paulownia tomentosa x fortunei* line TF 01 and *Paulownia elongata x fortunei* line EF 02, grown in hydroponic in response to salt stress

Treatments	Root dry mass [g]	Stem dry mass [g]	Leaf dry mass [g]	LAR [cm^2 g^{-1}]
Paulownia tomentosax fortunei				
Control	0.171±0.041b	0.205±0.011b	0.532±0.018b	497±52a
50 mM/l NaCl	0.051±0.002a	0.038±0.008a	0.117±0.016a	657±55ab
100 mM/l NaCl	0.067±0.003a	0.045±0.002a	0.119±0.023a	550±65a
200 mM/l NaCl	0.044±0.002a	0.031±0.003a	0.071±0.009a	794±34b
Paulownia elongata x fortunei				
Control	0.211±0.081b	0.280±0.012b	0.742±0.021b	415±54a
50 mM/l NaCl	0.180±0.013a	0.200±0.016a	0.430±0.042a	407±42a
100 mM/l NaCl	0.148±0.014a	0.222±0.027a	0.449±0.050a	341±15a
200 mM/l NaCl	0.140±0.017a	0.201±0.092a	0.540±0.021a	380±64a

Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.1).

The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen. In previous study we estrablished that MDA concentration is enhanced with increasing salinity levels and the maximum values are observed at 200 mM/l NaCl [9]. The results indicated that salt stress produced more reactive oxygen species, resulting in more increased lipid peroxidative products and oxidative stress in *Paulownia tomentosa x fortunei* line than in *Paulownia elongata x fortunei* line. The concentrations of H_2O_2 and total glutathione are enhanced, but that of reduced ascorbate declined in the leaves of TF 01. Hydrogen peroxide and glutathione concentrations increased in a different manner in the leaves of EF 02, while ascorbate concentration decreased compared to control. Highest concentrations of H_2O_2 and total glutathione are established at 50 mM /l NaCl (Table 2).

The level of the antioxidant enzymes, such as superoxide dismutase (SOD), GPOX and CAT may determine the sensitivity of plants to lipid peroxidation [7]. In our study, with increasing salinity levels, activities of the antioxidant enzymes CAT, GPOX and GR are enhanced by salt treatment of *Paulownia tomentosa x fortunei* plants, but CAT activity decreased in the leaves of *Paulownia elongata x fortunei* plants. CAT activity decreased at 200 mM/l NaCl after treatment of *Periploca sepium* Bunge seedlings [15], indicating that the ability of those antioxidant enzyme to eliminate oxygen species is limited. Highest GPOX, GR and APX activities are observed in *Paulownia elongata x fortunei* plants treated with 50 mM/l NaCl. The values decreased gradually with increasing salinity stress. The activities of GPOX, GR and APX are highest than that of CAT in both lines, which suggest that these enzymes provide a better defense mechanisms against salt stress – induced oxidative damage in *Paulownia tomentosa x fortunei* and *Paulownia elongata x fortunei* lines. The results are similar to that obtained for *Periploca sepium* Bunge seedlings [15].

Table 2. Mean values ± SD (n = 5-6) of hydrogen peroxide (H_2O_2), total glutathione (GSH+GSSG) and reduced ascorbate (Asc) contents in the leaves of *Paulownia tomentosa x fortunei* line TF 01 and *Paulownia elongata x fortunei* line EF 02, grown in hydroponic in response to salt stress

Treatments	H_2O_2	GSH+GSSG	Asc
	[nM g^{-1} FW]	[nM g^{-1} FW]	[nM g^{-1} FW]
Paulownia tomentosax fortunei			
Control	0.140±0.014a	4.67±0.21a	3.70±0.99b
50 mM/l NaCl	0.432±0.003b	12.05±0.47b	3.27±0.13ab
100 mM/l NaCl	0.501±0.014c	20.99±2.33c	2.83±0.99ab
200 mM/l NaCl	0.739±0.062d	27.39±2.43d	1.31±0.09a
Paulownia elongata x fortunei			
Control	0.162±0.011a	15.79±0.62ab	7.65±0.39d
50 mM/l NaCl	2.096±0.322c	30.25±5.25b	5.37±0.33c
100 mM/l NaCl	1.247±0.312b	18.27±1.08a	4.22±0.06b
200 mM/l NaCl	0.596±0.048a	17.02±1.06a	2.96±0.22

Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P<0.05$).

Table 3. Mean values ± SD (n = 5-6) of catalase (CAT), quiacol peroxidase (GPOX), glutathione reductase (GR) and ascorbate peroxidase (APX) activities in the leaves of *Paulownia tomentosa x fortunei* line TF 01 and *Paulownia elongata x fortunei* line EF 02, grown in hydroponic in response to salt stress

Treatments	CAT	GPOX	GR	APX
	[μM mg $pr.^{-1}$ min^{-1}]	[μM mg $pr.^{-1}$ min^{-1}]	[μM mg $pr.^{-1}$ min^{-1}]	[μM mg $pr.^{-1}$ min^{-1}]
Paulownia tomentosax fortunei				
Control	4.0±0.8a	5.7±0.1a	29.2±3.3a	32.2±1.5b
50 mM/l NaCl	6.6±0.2a	7.9±0.8a	33.9±3.8a	293.3±89.2ab
100 mM/l NaCl	8.5±0.3b	8.9±0.9a	45.8±3.8a	240.8±14.4ab
200 mM/l NaCl	14.7±1.7b	17.0±0.4b	139.9±29.6b	95.8±3.9a
Paulownia elongata x fortunei				
Control	24.6±0.8b	2.5±0.5a	36.3±3.7b	67.6±5.4a
50 mM/l NaCl	9.8±0.3a	20.8±1.4c	65.8±4.7a	367.3±58.7c
100 mM/l NaCl	4.5±0.2a	12.2±0.5b	39.8±3.5b	245.2±56.3b
200 mM/l NaCl	4.3±0.1a	10.6±0.3a	33.5±6.2a	306.0±13.9bc

Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P<0.05$).

In conclusion, *Paulownia elongata x fortunei* line possesses moderate salt tolerance capacity, which can help to adapt to 50 mM/l NaCl level. The results suggest a possibility to improve saline soil by utilizing *Paulownia*

tomentosa x fortunei line because it possesses better antioxidant defense and improves leaf area ratio (LAR) at 200 mM/l NaCl.

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