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Research Article

EFFECTS OF EDTA AND CITRATE ADDITION TO THE SOIL ON C4 PHOTOSYNTHETIC ENZYMES AND BIOCHEMICAL INDICATORS FOR HEAVY METAL TOLERANCE IN TWO PAULOWNIA HYBRIDS

Miladinova-Georgieva K.[1], M. Geneva[1*], Y. Markovska[2]

[1]Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 21,1113 Sofia, Bulgaria

[2]Faculty of Biology, University of Sofia, 8 Dragan Tsankov Bld., 1164 Sofia, Bulgaria

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Summary: Phytoremediation is a low cost, long term, environmentally and aesthetically friendly *in situ* technology for toxins removal from contaminated soils (especially heavy metals and metalloids), by the roots of plants with subsequent transport to aerial plant organs. This technology is suitable for large areas, in which other approaches would be expensive and ineffective. The influence of the addition of EDTA and citrate to heavy metal contaminated soil on the activities of C_4 photosynthetic enzymes, including phosphoenolpyruvate carboxylase; pyruvate, phosphate dikinase and NADP-malic enzyme in two-year-old plantlets of *Paulownia tomentosa* x *fortunei* – TF01 and *Paulownia elongata* x *fortunei* – EF02 was evaluated in a pot experiment. Treatment with 1 mM EDTA and 10 mM citrate had a protective effect against heavy metal stress through improvement of CO_2 concentration mechanism and total antioxidant activity in TF01, while the application of 1 mM EDTA and 5 mM citrate enhanced antioxidant activity only in EF02. Comparative analyses of results showed that *P. tomentosa* x *fortunei* – TF01 hybrid possessed a more effective CO_2 concentration mechanism and antioxidant defence under heavy metal stress than *P. elongata* x *fortunei* – EF02 and could be successfully used for phytoremediation of polluted soils.

Keywords: C_4 photosynthetic enzymes; citrate; EDTA; Paulownia; total antioxidant activity.

Abbreviations: $DPPH^{\cdot}$ – 2,2-diphenyl-1-picrylhydrazyl; EDTA – ethylendiaminetetraacetic acid; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDH – malate dehydrogenase; NADH – nicotinamide adenine dinucleotide; NADP – nicotinamide adenine dinucleotide phosphate; NADP-ME – NADP-malic enzyme; PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase; PPDK – pyruvate phosphate dikinase.

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*Corresponding author: boykova2@yahoo.com

INTRODUCTION

Heavy metals can accumulate in biological systems – humans, animals, microorganisms and plants, causing toxicity (D`Amore et al., 2005). Metal-contaminated soils are difficult to remediate and the used conventional engineering methods are expensive and non-effective (Zhou and Song, 2004). Phytoremediation (i.e. uptake and concentration of contaminants from the environment in plant biomass) is proposed as an alternative low cost technique to remediate soils contaminated with trace elements (Nouri et al., 2009; Kord et al., 2010).

Two directions have been suggested for phytoextraction of heavy metals, namely, natural phytoextraction, and chemically improved phytoextraction. The first one is based on utilization of the natural herbaceous hyperaccumulating plants characterized by slow growth to certain areas and highest metal accumulating capacity (Baker and Brooks, 1989). Low-biomass producing herbaceous accumulators, which accumulate only one specific element and possess low depth roots are widely used for phytoremediation of contaminated soils. The chemically improved phytoextraction known as induced phytoextraction is based on high yielding plants that extract a high amount of metals when their mobility in the soil has been increased by chemical treatments. Several chelating agents including organic acids (oxalic, citric, malic, succinic, tartaric, glutamic) and EDTA (ethylenediamine-tetraacetic acid), have been investigated for their ability to mobilize metals and increase the metal accumulation by different plant species (Wu et al., 1999; Madrid et al., 2002). Among these chelating agents, EDTA being a strong chelator for metals has been suggested to be most effective in mobility, solubility, and bioavailability in the soil solution phase, uptake by roots, and accumulation of soil-bound metals (Evangelou et al., 2007; Leleyter et al., 2012).

The deep contamination caused by a number of metals requires to use as an alternative fast-growing woody species with deep root system and the ability to grow on nutrient-poor soil. Some of them (poplar, willow, black locust, ash or alder) are successfully used for remediation of substrates contaminated with inorganic and organic pollutants (Pulford and Watson, 2003; Jensen et al., 2009; Castro-Rodríguez et al., 2016). Woody species from the genus *Paulownia* (Paulowniaceae) are native originating from China. *Paulownia tomentosa* has been introduced into Asia, USA, Australia and Europe as a high -yielding plant species (Woods, 2008). Over the last two decades *Paulownia* spp. have been extensively studied for phytoremediation purposes due to their tolerance to heavy metals in combination with outstanding growth rate and leaf expansion (Azzarello et al., 2012; Doumett et al., 2008; 2011; Stankovic et al., 2009; Wang et al., 2010).

In Bulgaria *Paulownia* spp. are micropropagated by the BIOTREE Company, according to the technology registered by Biotree Ltd. (Miladinova et al., 2013). Two hybrid lines (*P. tomentosa x fortunei* – TF01 and *P. elongata x fortunei* – EF02) have been

micropropagated and patented with the purpose of obtaining two types of plants: *(i)* short and branchy individuals and *(ii)* less branchy individuals for the purpose of wood material formation. The information about heavy metal tolerance of these hybrid lines and possibilities to be used as phytoremediators of contaminated soils is still insufficient (Tzvetkova et al., 2013; 2015).

Heavy metal stress reduces CO_2 availability in the leaves and inhibits carbon fixation with a consequence of growth reduction. Photosynthetic efficiency in C_3 plants (grown predominantly in temperate regions) depends on the activity of the primary carboxylating enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Lawlor and Tezara, 2009). CO_2 concentration mechanisms are evolved to reduce oxygenase activity of Rubisco when the environmental conditions are unfavourable (Sage, 2004). Paulownia is characterized as a tree with C_3 photosynthetic pathway (Ivanova et al., 2018). C_4 and CAM plants (grown predominantly in tropical and desert regions) are known to be more resistant to stress conditions than C_3 plants because CO_2 concentration mechanisms allow them to perform CO_2 fixation to phosphoenolpyruvate (PEP) via the secondary carboxylating enzyme phosphoenolpyruvate carboxylase (PEPC). The metabolic cycle is connected by a pyruvate, phosphate dikinase (PPDK) that catalyses the regeneration of PEP from pyruvate (Edwards et al., 2004; Sage, 2004). The major products of carbon assimilation pathways in most of the plants are sucrose and starch. Sugars are considered as important metabolites because they the first complex organic compounds formed in the plant as a result of photosynthesis, and they also provide a major source of respiratory energy. According to Anand and al. (2017) the heavy metals Pb, Cd, Hg and Zn greatly reduced the total soluble sugar and protein contents of *Azolla filiculoides*.

Intensive studies have been carried out on the evolution of the physiological role of C_4 metabolic enzymes in C_3 plants (Häusler et al., 2002). In C_3 plants, the content and activity levels of C_4 photosynthetic enzymes are lower compared with C_4 plants. Different hypothetic schemes for the possible CO_2 concentration mechanisms have been considered in C_3 plants under water, salt, low-temperature and heavy metal stress conditions (Fridliand and Kaler, 1988). It is suggested that PEP system functioning in higher plants becomes necessary when an organism is forced to reorganize its central metabolism for adaptation to changes in certain conditions and improvement of the metabolic conversions. Cd excess has a series of harmful effects, including inhibition of plant growth, disorder of nutrient uptake, inactivation of enzymes of carbon dioxide (CO_2) fixation and inhibition of photosynthesis (Duo et al., 2016). It has also been reported that Cd generates oxidative stress in plants through inducing the production of reactive oxygen species (ROS). To remove ROS, plants have evolved a series of antioxidant enzymatic and non-enzymatic systems. There is a lack of data about the relationship between C_4 photosynthetic enzyme activities and the antioxidant capacity of C_3 *Paulownia*

plants.

The aim of this research was to evaluate the influence of different concentrations of EDTA and citrate added to the soil on the activities of some C_4 photosynthetic enzymes including PEPC, PPDK and NADP-malic enzyme (NADP-ME) and reducing sugars in the leaves of *Paulownia tomentosa* x *fortunei* (TF01) and *Paulownia elongata* x *fortunei* (EF02) hybrids in order to elucidate their roles in plant adaptation to heavy metal stress. Changes in total antioxidant capacity in the leaves of both species after addition of chelating agents were also measured.

MATERIALS AND METHODS

Sampling site, plant materials and pot experiments

Two-year-old plantlets of *P. tomentosa x fortunei* – TF01 and *P. elongata x fortunei* – EF02, derived from *in vitro* micropropagation of seedlings (Miladinova et al., 2013), were cultivated in 50 plastic pots (one plants per plot) filled with soil aliquots with 2.5 kg dry mass, collected from the field near waste depository of Kremikovtzi ferrous metallurgical combine, Sofia, Bulgaria. The agrochemical soil characteristics were described by Tzvetkova et al. (2013). The soil was heavily polluted with Cd, because the content of Cd exceeded Bulgarian maximal permissible concentrations 1.6 times. All pots were adjusted daily by weight to 60% water holding capacity with tap water to maintain vigorous plant growth. The experiment was conducted in a glasshouse from the beginning of April 2014 to the end of July 2014. The glasshouse temperatures ranged from 15 to 35°C, and relative humidity varied between 40 and 65%. The plants were harvested at the end of July 2014.

The influence of the addition of complexing agents was evaluated using EDTA (ethylenediaminetetraacetic di-sodium salt dihydrate, purity ≥ 97%, Fluka) and citrate (citric trisodium salt dihydrate, purity ≥ 99%, Fluka), applied 42 days after planting (June 2014) at 1, 5 and 10 mM kg^{-1} soil dry mass, pH 8.00 (the natural pH of the soil). The complexing agents were applied to the soil in a single dose via manual dispersion of 150 ml of their aqueous solutions at concentrations of 0.04, 0.2 and 0.4 M. Non-treated pots were used as control. Thirty days after EDTA and citrate application plants (five replicates for each test) were harvested for analysis.

Determination of C_4 photosynthetic enzyme activities

Extraction procedures of PEPC and PPDK were performed according to Ashton et al. (1990). About 0.2 g of fresh material from the fourth fully developed leaves were harvested in the morning, ground into fine powder with liquid nitrogen, then 10 ml of extraction buffer containing 50 mM HEPES-KOH (pH 7.5) and 10 mM dithiothreitol (DTT) was added. For extraction of PEPC only, 5 mg ml^{-1} bovine serum albumin, 5 mM $MgCl_2$ and 2 mM K_3PO_4 were added. The mixtures were centrifuged at 10 000 x g at 4°C for 10 min and the supernatants were applied on Sephadex G-25 (Pharmacia) equilibrated with HEPES-KOH buffer.

The activity of PEPC (EC 4.1.1.31) was assayed at 340 nm by following the reduction of oxaloacetate by nicotinamide adenine dinucleotide (NADH) in the

presence of malate dehydrogenase (MDH) using UV-VIS spectrophotometer Boeco S-22 (Germany). Reaction mixture (2 ml) contained 25 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$, 2 mM DTT, 1 mM $NaHCO_3$, 5 mM glucose-6-phosphate, 5 mM PEP, 0.2 mM NADH, 2 U of MDH and 0.2 ml of the enzyme extract. The decrease in absorbance due to the oxidation of NADH was measured for 3 min (Ashton et al., 1990).

The activity assay of PPDK (EC 2.7.9.1) was performed at 340 nm in the forward direction by coupling the production of PEP to NADH via PEPC and MDH (Ashton et al., 1990). Reaction mixture (2 ml) contained 25 mM HEPES-KOH буфер (pH 8.0), 8 mM $MgSO_4$, 10 mM DTT, 10 mM $NaHCO_3$, 2 mM pyruvate, 5 mM $(NH_4)_2SO_4$, 1 mM glucose-6-phosphate, 1 mM ATP, 2.5 mM K_3PO_4, 0.2 mM NADH, 0.5 U of PEPC, 2 U of MDH and 0.2 ml enzyme extract. The contents were allowed to react at 30°C and the decrease in absorbance due to the oxidation of NADH was measured for 3 min.

The activity assay of NADP-ME (EC 1.1.1.40) was performed spectrophotometrically at 340 nm by following the reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the presence of malate (Garnier–Dardart and Quieroz, 1974). The reaction mixture (2 ml) contained 50 mM HEPES-KOH (pH 7.5), 3 mM $MgCl_2$, 0.25 mM NADP, 3 mM malate and 0.1 ml enzyme extract. The increase in absorbance due to the reduction of NADP was measured for 3 min.

The determination of protein content was performed by the method of Lowry et al. (1951).

Reducing sugars assay

Dry leaf material (0.50 g) was ground in a mortar with 5.0 ml 80% ethanol. The homogenous mixture obtained was allowed to stay for 20 min at room temperature, followed by filtration through Whatman qualitative filter paper, Grade 4. The total content of reducing sugars was determined in the ethanol extracts by using the phenol/sulphuric acid method of Dubois et al. (1956). An aliquot of 0.25 ml of the filtrate was added and mixed with 0.75 ml distilled water, 0.5 ml 5% phenol and 2.5 ml 98% H_2SO_4. The resulting orange complex was then measured at 485 nm after 1 h with intermittent shaking. The content of reducing sugars was expressed as glucose equivalents in mg g^{-1} of the sample. All values were expressed as mg of glucose equivalents per 1 g DW.

DPPH assay

Free radicals scavenging activity was measured from the bleaching of the purple-coloured methanol solution of free stable radical (2,2-diphenyl-1-picrylhydrazyl, $DPPH^{\cdot}$) inhibition after Tepe et al. (2006). $DPPH^{\cdot}$ radical is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The inhibition of free radical $DPPH^{\cdot}$ in percent (I %) was calculated in the following way:

$$I\% = (A_{blank} - A_{sampe} / A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound, i.e. plant extracts. The reaction mixture consisted of different concentrations from 15 to 180 μg ml^{-1} plant methanol extract, 2.4 ml methanol and 0.1 mM methanol solution

of DPPH˙. Control and tested samples were incubated in the dark for 30 min before spectrophotometric assay.

Statistical analysis

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

RESULTS

The 10 mM EDTA concentration is usually reported in the literature as toxic for many herbaceous species (Wu et al., 1999). Surprisingly, Doumett et al. (2008) did not find any phytotoxic symptoms after 10 mM EDTA treatment of *Paulownia tomentosa* and concluded that this plant possessed strong resistance towards the presence of high chelator concentrations in the soil. Our results showed that application of 10 mM EDTA was harmful to both hybrid lines, in which leaves presented an extensive chlorosis and necrotic areas, and most of the plants died within a few days after EDTA application (Fig. 1). Application of EDTA at 1 and 5mM did not effect negatively plant growth. Plants treated with lower EDTA concentrations and 1, 5, and 10 mM citrate did not show any phytotoxic symptoms upon visual assessment, they had the same appearance as control plants.

The results showed that EF02 control leaves possessed 4-times higher PEPC activity as compared to the TF01control (Fig. 2A). A dramatic decrease in PEPC activity in EF02 leaves after all treatments applied was observed, while in TF01 a significant enhancement was established after treatment with 1 mM EDTA and 1 mM and 10 mM citrate. All treatments led to a decline in PPDK activity in TF01 leaves in comparison with the control as the strongest impact was observed under treatments with 5 mM EDTA and 5 mM citrate, respectively (Fig. 2B). Treatment with 1 mM EDTA and 10 mM citrate caused an increase in PPDK activity in EF02 leaves. NADP-ME activity did not change in TF01 after treatment with 5 mM EDTA and 5 mM citrate, while in EF02 a significant decrease was established after all treatments as compared to control. Lower concentrations of the

Figure 1. *Paulownia tomentosa* x *fortunei* and *Paulownia elongata* x *fortunei* grown in heavy metal polluted soil on the third day after application of 10 mM EDTA.

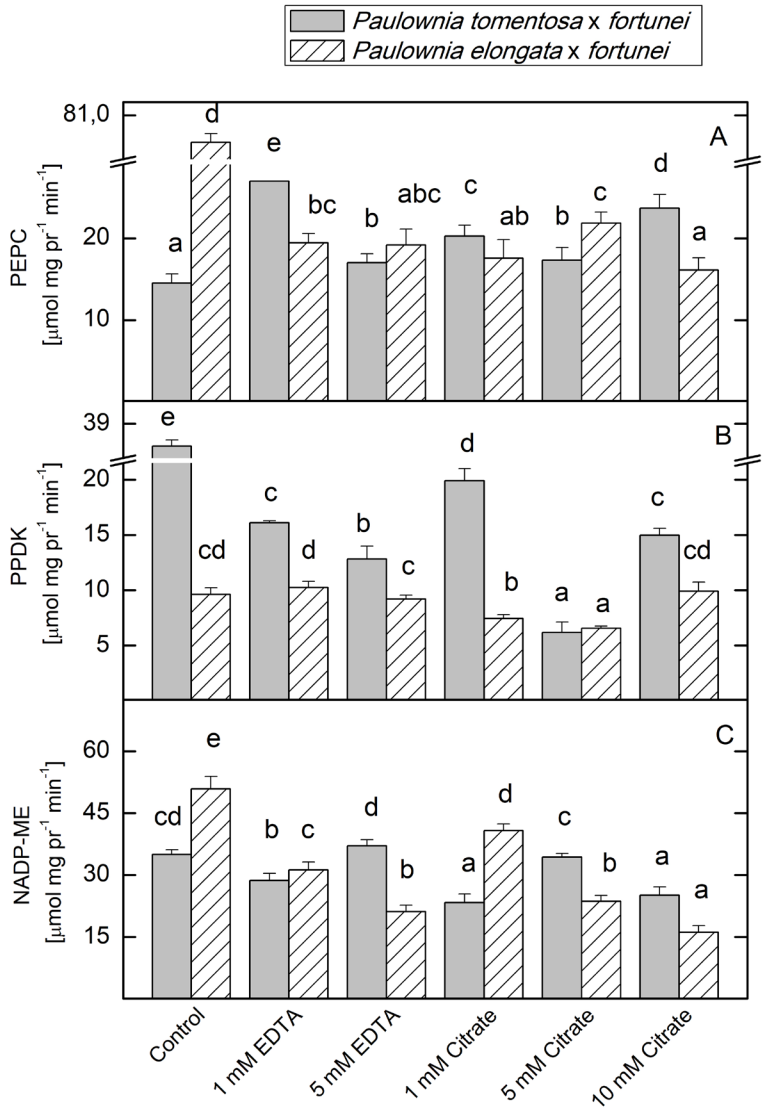

Figure 2. Changes in phosphoenolpyruvate carboxylase (PEPC - A), pyruvate, phosphate dikinase (PPDK - B) and NADP-malic enzyme (NADP-ME - C) activities at the end of the experiment in mature leaves of *Paulownia tomentosa* x *fortunei* (TF01) and *Paulownia elongata* x *fortunei* (EF02) lines grown in heavy metal polluted soil supplemented with EDTA and citrate as complexing agents. Mean values ± SE (n = 3-5). Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P<0.05$).

chelating agents applied (1 mM) increased negligibly these values (Fig. 2C).

Soluble sugars prevailed in EF02 control leaves as compared to TF01 control. The highest values were measured in control plants of both hybrid lines. The lowest value was established after application of the lowest concentrations of the chelating agents (1 mM EDTA) (Fig. 3).

Higher values of the total antioxidant activity were established after all

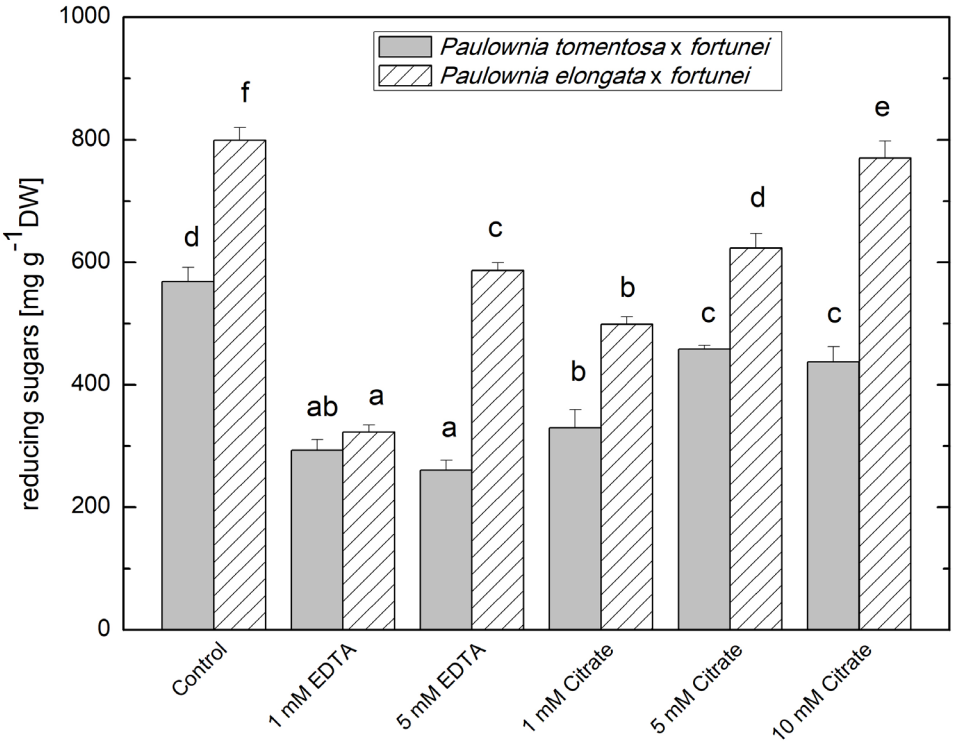

Figure 3. Changes in reducing sugars content at the end of the experiment in mature leaves of *Paulownia tomentosa* x *fortunei* (TF01) and *Paulownia elongata* x *fortunei* (EF02) lines grown in heavy metal polluted soil supplemented with EDTA and citrate as complexing agents. Mean values ± SE (n = 3-5). Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.05).

treatments in both TF01 and EF02 plants. No significant differences were established for the antioxidant capacity in leaves of TF01 hybrids treated with the two complexing agents added at different concentrations (Fig. 4). The antioxidant capacity was lower when EF02 plants were treated with 5 mM EDTA and 1 mM citrate in comparison with plants treated with the rest test concentrations.

DISCUSSION

Tzvetkova et al. (2015) have reported that two hybrid lines *P. tomentosa* x *fortunei* – TF01 and *P. elongata* x *fortunei* – EF02 are accumulators of Cu, Zn and Cd and can be used for phytoremediation of heavy metal contaminated soils. TF01 plants accumulated higher amounts of Pb and Zn in the aboveground parts, while EF02 plants – only Zn. Bahri et al (2015) confirmed that *Paulownia tomentosa* (Thunb.) Steud could be used in the phytoextraction of Zn and Pb from contaminated soils, but heavy metals caused slight reductions in all growth parameters and deterioration of photosynthetic performance. Miladinova-Georgieva et al. (2018) reported that net-photosynthesis was enhanced in the leaves of TF01 and EF02 upon application of 1 mM EDTA, 1 mM and 10 mM citrate. The obtained data showed

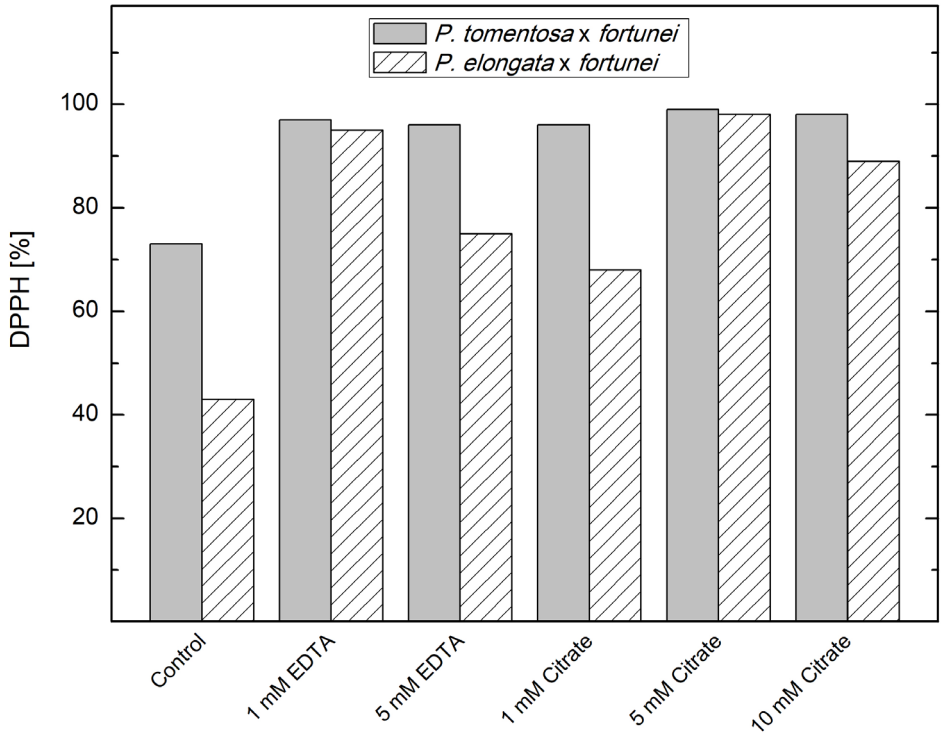

Figure 4. Changes in total antioxidant activity, measured by the DPPH method at the end of the experiment in mature leaves of *Paulownia tomentosa* x *fortunei* (TF01) and *Paulownia elongata* x *fortunei* (EF02) lines grown in heavy metal polluted soil supplemented with EDTA and citrate as complexing agents. Mean values ± SE (n = 3).

that both lines responded differently in terms of photosynthetic enzyme activities, soluble sugar levels and total antioxidant activity during treatments with increasing concentrations of chelating agents. TF01 plants reacted with increasing activities of PEPC, PPDK and total antioxidant activity at 1 mM EDTA, 1 mM and 10 mM citrate (Fig. 2A, B and Fig. 4). The highest values for PEPC, NADP-ME activities and soluble sugar content were established in control EF02 plants, but these values declined in a different manner after the treatments applied (Fig. 2A, C and Fig. 3). Total antioxidant activity was enhanced to a higher extent after treatment of EF02 with 1 mM EDTA and 5 or 10 mM citrate (Fig. 4).

C_4 plants differ from C_3 plants under the CO_2 concentrating mechanism, which has advantages in climatic conditions unfavourable for growth conditions such as high temperatures, low water availability, high irradiation or heavy metal soil contamination (Sage, 2004). The activities of PEPC, NADP-ME and PPDK, which participate in the process of concentrating CO_2, are enhanced in plants with the two types of photosynthesis under stress conditions (Doubnerová and Ryšlavá, 2011). Our results showed that control EF02 plants possessed higher PEPC, NADP-ME activities and soluble sugar content as compared to TF01 control plants (Fig. 2A, B and Fig. 4). PEPC and NADP-ME play a more pronounced role in

the synthesis and degradation of C_4 organic acids and participate in the process of CO_2 concentration, namely in EF02 control plants than in TF01. The increase in the C_4 cycle enzyme activities (PEPC) caused by heavy metals leads to an enhancement of the C_4 acid synthesis (previously malate – data not shown). CO_2 involved as a result of the subsequent decarboxylation of this acid by NADP-ME is used in the Calvin cycle as a substrate for Rubisco, providing the normal performance of the reactions in the cycle. These results showed that the studied enzymes are actively involved in the process of biochemical adaptation against the effects of heavy metals in EF02 control plants. PEPC and NADP-ME activities have undergone an induction that confirmed their key roles in the biosynthesis and degradation of dicarbonic acids. Because PEPC activity changed less in TF01 than in EF02, probably non-carbohydrate pathways of the C-photosynthetic metabolism remain more stable under heavy metal stress. It is known that soluble sugar production in the leaves of Paulownia rise at warm conditions (Woods, 2008). Our results showed that EF02 control plants accumulated more soluble sugars than TF01 plants (Fig. 3). The accumulation of sucrose could potentially play a direct role in osmoregulation and could also provide quickly metabolizable carbohydrates for energy production when carbon is diverted from growth to other functions (Hare et al., 1998). According to Miladinova-Georgieva et al. (2018) 1 mM EDTA and 10 mM citrate added to the soil enhanced leaf growth (the highest values for total leaf area/total dry mass ratio, total leaf area/leaf number ratio and rate of net-photosynthesis). It would be helpful for TF01 to remediate metal contaminated soils because improvement of the photosynthetic surface under stress conditions is critical to the optimum performance of phytoremediation.

A reduction of reactive oxygen species after EDTA treatment in the heavy metal accumulating species *Sedum alfredii* L. was established (Huang et al., 2008). EDTA reduced heavy metal impact, the activities of POX, CAT, enzymes of the ascorbate-glutathione cycle, but the levels of ascorbate, reduced glutathione and flavonoids were enhanced in the shoots of *Tribulus terrestris* (Markovska et al., 2013). Because citrate is a natural molecule, it can be metabolized by plants more easily than EDTA. The use of natural, rapidly biodegradable ligands at much higher concentrations and with a regular renewal of the complexing agent application will lead to an improvement of plant metal accumulation without increasing environmental impact. The activities of CAT, POX, GR and APX in the leaves of TF01 were enhanced after treatment with increasing concentrations of EDTA, while in EF02 they were decreased (Miladinova - Georgieva et al., 2017). In our experiments, however, we did not establish any significant changes in the total antioxidant activity in the leaves of TF01 after treatment with EDTA and citrate at all concentrations applied. Total antioxidant activity in the leaves of EF02 plants decreased with increasing EDTA concentration in the soil, but it enhanced with increasing citrate concentration. There is no clear correlation between the changes of antioxidant enzyme activities and total antioxidant capacity measured after EDTA and citrate application in both plants.

CONCLUSION

The selected two *Paulownia* lines (*P. tomentosa x fortunei* – TF01 and *P. elongata x fortunei* – EF02) are promising species for phytoremediation of heavy metal contaminated soils due mainly to very high biomass productivity rather than metal accumulation potential. In agreement with the current status of research on induced phytoremediation, we conclude that the application of 1 mM EDTA and 10 mM citrate was more effective for improvement of CO_2 concentration mechanism and total antioxidant activity in *Paulownia tomentosa* x *fortunei*, while the application of 1 mM EDTA and 5 mM citrate enhanced the antioxidant activity only in *Paulownia elongata* x *fortunei*. The parameters studied may be used as reliable indicators for the investigation of tolerance mechanisms in these lines.

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