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A POSSIBLE ROLE OF C4 PHOTOSYNTHETIC ENZYMES IN TOLERANCE OF TWO *PAULOWNIA* HYBRID LINES TO SALINITY

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Abstract: The effect of salinity on the activities of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), NADP-malate dehydrogenase (NADP-MDH, EC 1.1.1.82) and NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37) in three years-aged *Paulownia* plants (*P. tomentosa x fortunei* - TF and *P. elongata x elongata* - T4) grown on two soils differing in salinity for a period of four months was investigated. The soil samples were collected from two areas of the village Belozem near the city of Plovdiv, Bulgaria. It was found that increase of the soil salinity leads to a more pronounced enhance of the activity of PEPC and malate content in the sensitive line TF at the early stages of vegetation. The activities of investigated enzymes declined significantly in T4 at the end of vegetation. The K/Na ratio in leaves of the TF line increased gradually during vegetation, while this parameter was higher in the leaves of the T4 line in the middle stage of the vegetation. Increases in the enzyme activities in both lines suggested that they might be playing a different role in their tolerance to salinity. T4 is more tolerant to salt stress than TF because it has improved growth parameters - stem length, leaf number, total dry biomass and total leaf area at the end of vegetation.

INTRODUCTION

Natural or primary salinity is more widespread in arid and semi-arid regions of the world and results from the accumulation of soluble salts in soils or groundwater over long geological periods. Besides the naturally-formed saline and sodic soils, the occurrence of so-called secondary salt – affected soils is due to the application of different agricultural practices and continues to expand (Munns, 2011).

Deeper soil pollution and salinity require the application of an alternative fast-growing woody species with a deep root system and the ability to grow on nutrient-poor soils. Some of them (poplar, willow, black locust, ash, alder and paulownia) are successfully used for remediation of substrates, contaminated by inorganic (or organic) pollutants. Munns (2011) proposed that increased salt tolerance of perennial species (such as woody species) used for fodder or fuel production is 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. Areas of degraded soils can be reduced by planting salt tolerant species and their purpose can be switched to the cultivation of high-yielding plants producing woody biomass, biofuel or economic important bioactive products.

The woody species from the genus *Paulownia* (Paulowniaceae) are native to China. *Paulownia tomentosa* has been introduced into Asia, USA, Australia and Europe as a high-yielding plant (Woods, 2008). These trees can be used for the production of energy, paper pulp and wooden building materials. The combination between deep root system and high growth rate explain the use of *Paulownia* plants for bioremediation of polluted and degraded soils.

Influence of salinity on a key metabolic process in plants – photosynthesis – is direct (by limiting stomatal conductivity and decreasing biochemical capacity of mesophyll cells to photosynthetic fixation of CO_2) or secondary, such as oxidative stress arising from the superimposition of multiple stresses (Chaves et al., 2009). Carbon balance under stress conditions is determined from its duration and acquiring positive values depends on the rate and degree of photosynthetic recovery.

Photosynthetic efficiency of C_3 plants (to which *Paulownia* hybrid lines belong) depends to a great extent on the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the possibilities to reduce photorespiration (oxygenase activity of Rubisco) (Sage, 2004). C_4 plants differ from C_3 plants by their CO_2 concentrating mechanism, which have advantages in extreme growth conditions such as high temperatures, low water availability, high irradiation or saline soils (Edwards et al., 2004). Phosphoenolpyruvate carbolylase (PEPC), NADP-malate dehydrogenase (NADP-MDH) and NAD-malate dehydrogenase (NAD-MDH) participate in the process of CO_2 concentrating in C_4 photosynthesis. The activities of all these enzymes are enhanced in plants with two types of photosynthesis under stress conditions (Doubnerova et al., 2011; Babaev et al., 2013).

In the present study, pot experiments were carried out to investigate the differences in the leaf K/Na ratio between *P. tomentosa x fortunei* – TF and *P. elongata x elongata* - T4 plants grown on two types of saline soils for a period of four months. The dynamics of the changes of activities of C_4 photosynthetic enzymes, including PEPC, NADP-MDH and NAD-MDH were traced in order to elucidate their role in adaptation of *Paulownia* lines differing in salt tolerance.

MATERIALS AND METHODS

Three-year-old plantlets of *P. tomentosa x fortunei* – TF and *P. elongata x elongata* - T4, derived from *in vitro* micropropagation of seedlings, were cultivated in plastic pots filled with soil aliquots of 2.5 kg dry weight, collected from two different areas situated in the vicinity of the village Belozem near Plovdiv city, Bulgaria. On the basis of agrochemical characteristics the first type of soil was characterized as non-saline and the second type of soil was middle alkaline. The second soil possessed exchangeable Na content, about 5.3 times higher and sodium adsorption ratio (SAR) about 6 times higher in comparison to the first soil. The electrical conductivity of the saline soil was 14.0 mS/cm, while that of the non-saline was 6.3 mS/cm. The pH values of both soils were higher than 8.00 (Ivanova et al., 2015). The experiment was conducted in a glasshouse, supplied with natural sunlight from 20th April to 7th October, 2014. The glasshouse temperatures varied from 15^{o}C to 35^{o}C, and relative humidity ranged from 40% to 65%.

Enzyme assays

The plant samples from fully developed leaves were harvested on the day of the experiment after 2-3 h of sunlight exposure four times in the period of vegetation. The homogenization of 0.5 g fresh material was carried out in a medium of 0.1 M Tris-HCl (pH 8.3), containing 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol and 1% polyvinylpyrrolidone (soluble) as described by Babaev et al. (2013). The homogenates were squeezed through 4 layers of cloth and centrifuged at 300 g for 5 min and at 1000 g for 20 min. The supernatants were used for the determination of the enzyme activities.

All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at λ=340 nm (27^{o} C) using UV-VIS spectrophotometer (Boeco S22, Germany) in a 3 ml reaction mixture as described below. In each case the reaction was initiated by the compound listed last. The assay conditions for all enzymes tested were modified from the referred sources to give optimum activities in leaf extracts from *Paulownia* plants.

PEPC (EC 4.1.1.31): 50 mM Tris-HCl buffer (pH 8.5), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.25 mM NADH, 10 U malate dehydrogenase, 10 mM phosphoenolpyruvate (PEP), 50 µl of the enzyme preparation. The reaction was initiated by adding PEP (Romanova, 1980);

NADP-MDH (EC 1.1.1.82): Before the determination NADP-MDH was activated in the presence of 2-dithiothreitol (DTT) for 15 min. Then the total NADP-MDH was determined. The reaction mixture consisted of 100 mM Tris-HCl buffer (pH 8.0), 10 mg ml^{-1} albumin (BSA), 20 mM $MgCl_2$, 10 mM NADPH, 10 mM oxaloacetate (OAA), 50 µl of the enzyme preparation. The reaction was initiated by adding OAA (Scheibe and Stitt, 1988);

NAD-MDH (EC 1.1.1.37): 100 mM Tris-HCl buffer (pH 8.0), 15 mM OAA, 12 mM NADH, 50 µl of the enzyme preparation. The reaction was initiated by adding NADH (Scheibe and Stitt, 1988).

Quantitative determination of protein was carried out according to Lowry et al. (1951).

Malate assay

100 mg lyophilized leaf material was homogenized in a medium of 5 ml 1N $HClO_4$ at room temperature for 30 min. The samples were centrifuged at 10 000 g for 5 min. The supernatants were neutralized with 5 M K_2CO_3 and cooled in ice bath. The reaction mixture for determination of malate consisted of 2.5 ml hydrazine-glycine buffer (pH 9.0), 10 mM NAD and 5 µl malate dehydrogenase (5 mg protein ml^{-1}). The reaction was initiated by adding 200 µl supernatant and the absorbance is traced at λ=340 nm (27° C) for 5 min (Gutmann and Wahlefeld, 1974).

Ion content

To determine the content of K^+ and Na^+ (mg g DW^{-1}), 0.25 g dry leaf samples were extracted after acidic digestion with Suprapur grade Fluka reagents and analyzed by atomic absorption spectrophotometer (Perkin-Elmer 5000, UK). The K^+/Na^+ ratio was calculated from the content of K^+ and Na^+.

Reagents

All solvents were of analytical grade. The chemicals used for enzyme activity measurement were obtained from Sigma – Aldrich (United States) and Fluka Chemie AG (Switzerland). All other chemicals were of analytical grade.

Statistical data analysis

Data are expressed as means ±SE. Comparison of means was performed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis (Statgraphics Plus, v. 2.1).

RESULTS AND DISCUSSION

Intensive investigations 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 compared to C_4 plants, the content and activity levels of C_4 photosynthetic enzymes are lower. Different hypothetical schemes for the possible CO_2 concentrating mechanism have been considered in C_3 plants under water, salt and low temperature stress conditions (Fridliand and Kaler, 1988; Kaiser, 1987). It is suggested that phosphoenolpyruvate (PEP) system functioning in higher plants becomes necessary when an organism is forced to reorganize its basic

metabolism for adaptation to changes of certain conditions and improvement of the metabolic conversions.

Activities of PEPC, NADP-MDH and NAD-MDH have been investigated in fully developed leaves of two *Paulownia* lines grown on non-saline and saline soils. Our preliminary investigations showed that T4 is more resistant to salinity because its growth and gas exchange characteristics are less affected in comparison to TF. The latter exhibited much better response in terms of antioxidant enzyme activities (Ivanova et al., 2015).

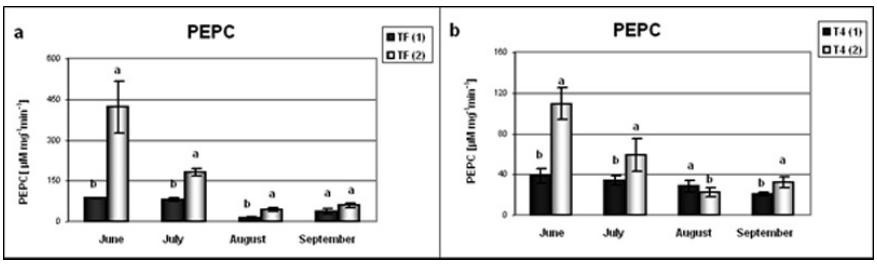

Figure 1. Changes in PEPC activities in the leaves of two hybrid lines *P. tomentosa x fortunei* – TF (a) and *P. elongata x elongata* - T4 (b) grown on non-saline (1) and saline (2) soils during vegetative period.

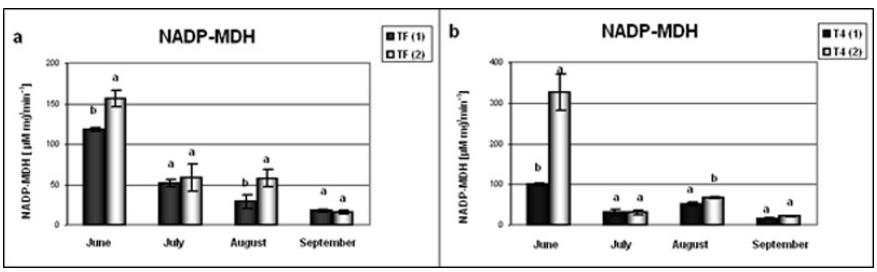

Figure 2. Changes in NADP-MDH activities in the leaves of two hybrid lines *P. tomentosa x fortunei* – TF (a) and *P. elongata x elongata* - T4 (b) grown on non-saline (1) and saline (2) soils during vegetative period.

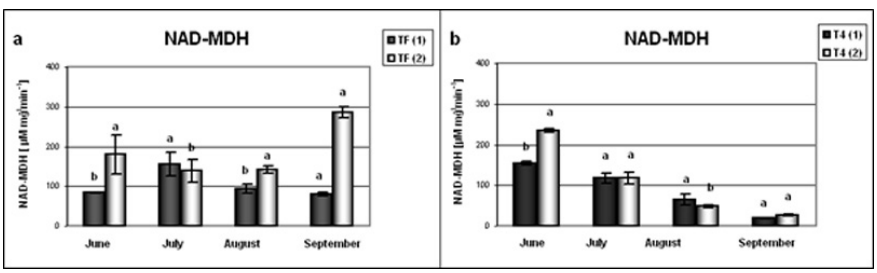

Figure 3. Changes in NAD-MDH activities in the leaves of two hybrid lines *P. tomentosa x fortunei* – TF (a) and *P. elongata x elongata* - T4 (b) grown on non-saline (1) and saline (2) soils during vegetative period.

As shown in Fig. 1a,b, 2a,b and 3a,b, the activities of PEPC, NADP-MDH and NAD-MDH initially increased and then decreased gradually by the end of the vegetation, except for NAD-MDH in TF (Fig. 3a). Under saline conditions, activities of all investigated enzymes increased more rapidly namely in TF. Malate content showed the same manner of changes in the leaves of both lines, but average values obtained in T4 grown on non-saline soil in June and August are higher as compared to the saline soil (Fig. 4a and Fig. 4b). It is known that PEPC activity increased under stress conditions and the susceptibility of enzyme to the allosteric inhibitor malate decreased (Foyer et al., 1998). Higher values of PEPC, NAD-MDH activities and malate content proved that this metabolite is formed predominantly with the participation of PEPC and NAD-MDH in TF, while in T4 NADP-MDH prevailed. Malate can function as a vascular osmolyte and may also serve as an additional sink for carbon assimilation and reducing equivalents (Crecelius et al., 2003). Bouraima et al. (1987) concluded that PEPC and MDH may be used as an effective marker of salt tolerance in C_4 plants, because PEPC activity is enhanced in salt-tolerant genotype and decreased in the most sensitive one. Nemat Alla et al. (2011) reported that NaCl doubled PEPC protein in *Atriplex humilis*, while Rubisco is unchanged. They concluded that *A. humilis* tolerates NaCl treatment through decreasing growth and saving the photosynthetic enzymes, particularly PEPC. On the contrary, Marques Da Silva and Arrabaca (2004) found that rapid stress increased Rubisco activation in *Setaria sphacelana*, while PEPC showed a substantial decrease of activity.

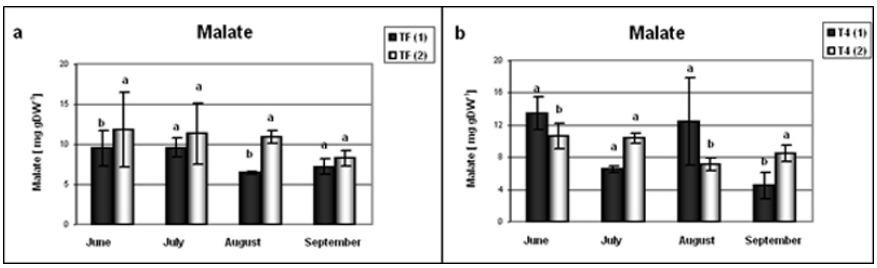

Figure 4. Changes in malate contents in the leaves of two hybrid lines *P. tomentosa x fortunei* – TF (a) and *P. elongata x elongata* - T4 (b) grown on non-saline (1) and saline (2) soils during vegetative period.

The activity of NAD-MDH in the leaves of TF grown on saline soil is highest at the end of the vegetation (Fig. 3a), while NADP-MDH activity is highest at the beginning of this period in both lines (Fig. 2a and Fig. 2b). NADP-MDH is light-regulated enzyme and it is located in chloroplasts (Scheibe and Stitt, 1988). PEPC activity as well as malate content were highest in the leaves of TF in June and July (Fig. 1a and Fig. 4a). The increase in the C_4 cycle enzyme activities caused by salinity led to the enhancement of the C_4 acid synthesis (malate). CO_2 released

as a result of the subsequent decarboxylation of these acids is used in the Calvin cycle as a substrate for Rubisco, providing optimal 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 salt. PEPC and NAD-MDH activities are the most subjected to induction that confirmed their key roles in the biosynthesis of dicarboxylic acids. Because PEPC activity changed less in T4 than in TF probably non-carbohydrate pathways of the C-photosynthetic metabolism remain more stable under salt stress. It is known that, contrary to Rubisco, PEPC does not require ATP and NADPH, through the latter is necessary for the reduction in the triosephosphates production. Moreover, PEPC compared to Rubisco has more affinity for C-acids and therefore its activity decreases less when the stomata are closed under stress conditions.

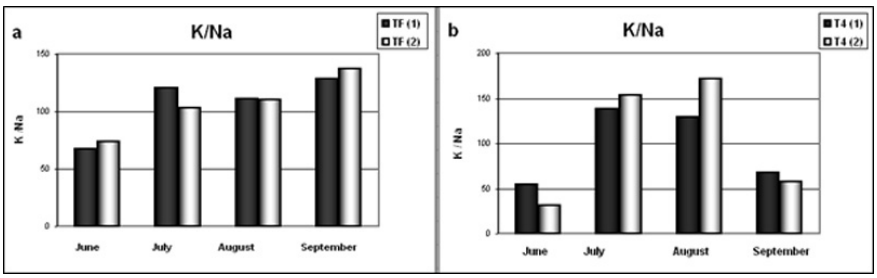

Figure 5. Changes in K/Na ratio in the leaves of two hybrid lines *P. tomentosa x fortunei* – TF (a) and *P. elongata x elongata* - T4 (b) grown on non-saline (1) and saline (2) soils during vegetative period.

The K/Na ratio in the leaves of TF increased gradually during vegetation, while this parameter was higher in the leaves of T4 in the middle stages of the vegetation. The values obtained for both lines grown on saline soil are higher, except for these, measured at the beginning and the end of vegetation of T4 (Fig. 5a,b).This ratio is an important selection criterion for salt tolerance (Ashraf and Orooj, 2006) and highest values determined more salt tolerant plant species (Erdal and Çakirlar, 2014). The dynamics of the changes between PEPC, NADP-MDH, NAD-MDH and K/Na ratio in the leaves of TF is opposite, while in T4 they are not regular. The balance between water flow (sum of water accumulation and transpiration) and the increase in the amounts of nutrients or favorable nutrient ratio e.g. K/Na are important factors for improvement of leaf elongation and plant growth. Transpiration rate is more enhanced in T4 grown on saline soil at the end of the vegetation period as compared to TF (Ivanova et al., 2015). That is why,T4 improved its growth parameters - stem length, leaf number, total dry biomass and total leaf area (data not shown).

CONCLUSIONS

Under saline conditions, activities of investigated enzymes - PEPC, NAD- and NADP-MDH in both plants are higher at the beginning of vegetation in comparison with the end of this period. The increase in the C_4 cycle enzyme activities caused by salinity led to the enhancement of the synthesis of malate. PEPC and NAD-MDH play more pronounced role in the synthesis of malate in TF, while in T4 NADP-MDH is important.

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