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Response of dihaploid tobacco roots to salt stress

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Abstract – Salinity is a common abiotic factor that affects plant growth and development. Seedlings of tobacco (*Nicotiana tabacum* L.) F1 hybrid DH10 and three dihaploid lines (207B, 238C and 239K) obtained by diploidization of anther-derived haploids of hybrid DH10 were subjected to 0, 100 and 200 mM NaCl in *in vitro* conditions for 33 days and the effect on roots was evaluated. In all lines and in the hybrid DH10 exposed to 200 mM NaCl evident root growth inhibition and increased proline content were noticed. However, in some cases lines differed in the activity of antioxidative enzymes, which could account for differences in their salinity tolerance. Increased activity of catalase and peroxidase in roots of line 239K could contribute to the more pronounced salinity tolerance previously reported for shoots of this line.

Keywords: antioxidative enzymes, *Nicotiana tabacum* L., proline, root, salinity, tobacco dihaploid lines

Introduction

Salinity is one of the most adverse environmental factors that reduce the growth and productivity of agricultural crops (Horie et al. 2012). In nature, it often comes together with drought and heat and all these stress factors may cause changes in plant water status, decreased activity and denaturation of structural proteins and leakiness of membranes caused by phospholipid bilayer disruption (Wang et al. 2003, Mahajan and Tuteja 2005).

Generally, the detrimental effects of salinity in plants are characterized by a decrease in growth rate, a change in root/shoot ratio and development of chlorosis (Parida and Das 2005). Reduced growth mainly occurs due to low water potential in the soil. In such conditions the possibility of cell dehydration is increased due to difficulty in the acquisition of water by plant roots. In addition, increased concentration of Na^+ and Cl^- in plant organs disturbs the intake of essential microelements such as K^+, leading to altered K^+/Na^+ ratios and disruption of cellular homeostasis (Apse and Blumwald 2007, Munns and Tester 2008).

In order to adjust osmotic potential, plants accumulate compatible osmolytes. The most common among them is the amino acid proline, which is broadly present in many organisms. Besides being an osmolyte, proline acts as a molecular chaperone: it enhances the activities of the enzymes, controls plant development and acts as a signal molecule. Proline also shows antioxidant property through reactive oxygen species (ROS) scavenging activity (Szèkely et al. 2008, Szábados and Savourè 2009).

Under the conditions of increased salinity, the levels of ROS can dramatically increase. In order to deal with these highly reactive species, antioxidative enzymes such as catalase (CAT), non-specific peroxidase (POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX) cooperate together to mitigate cellular damage such as unspecific oxidation of proteins, membrane lipids and nucleic acids. SOD converts superoxide radical ($O_2^{\bullet -}$) into oxygen and hydrogen peroxide while POD and CAT are involved in H_2O_2 detoxification (Bandeoğlu et al. 2004, Parvanova et al. 2004). Numerous studies emphasize that the antioxidative response strongly correlates with susceptibility and resistance to increased salinity (Acar et al. 2001, Tűrkan et al. 2005).

In previous work we investigated the response of shoots of F1 hybrid DH10 and four dihaploid tobacco lines derived from that hybrid (207B, 238C, 239K, 244B) to 100 and 200 mM NaCl. F1 hybrid and dihaploid lines were previously proved to be tolerant to potato virus Y (PVY) (Šmalcelj and Ćurković-Perica 2000). Salt stress caused growth retardation and induced proline and sodium accumulation in shoots of all dihaploids and hybrid DH10. In line 239K, salt induced higher activities of SOD, CAT and POD. Our results revealed that lines 238C and 244B were more susceptible than lines 207B and 239K to increased salinity, suggesting that tolerance to the virus is not associated with salinity tolerance (Marček et al. 2014). Although the roots are the first to encounter excess salinity and are potentially the first sites of damage or "line of defence" (Rewald et al. 2013), most studies of plant tolerance to high salinity

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focus on traits in the aboveground tissues; data on the phenotypical and physiological plasticity of root systems under salt stress are rare. Except having a role in the uptake of water and nutrients, roots also act as a sensory system, integrating changes in nutrient availability, water content and salinity in order to adjust root morphology for better exploitation of available resources (Gruber et al. 2013). Therefore, the purpose of this study was to expand our previous research by investigating the effect of NaCl on roots of dihaploid tobacco lines and hybrid DH10 in order to reveal if root responses to salinity may contribute to the salt tolerance of investigated tobacco lines.

Materials and methods

Plant material and treatments

Tobacco (*Nicotiana tabacum* L.) F1 hybrid DH10 (hybrid of cv. Virginia D and line GV3) and four dihaploid lines (207B, 238C, 239K, 244B) obtained by diploidization of anther-derived haploids of the hybrid DH10 (Šmalcelj and Ćurković-Perica 2000) were tested for salinity tolerance. Seeds, provided by the Duhanski Institut Zagreb (Croatia), were grown in pots containing a mixture of peat-sand (2:1). Ten weeks after planting, the seedlings were surface sterilised in 70% ethanol for 10–15 s and then for 15 min in 1% (w/v) sodium hypochlorite (NaOCl) supplemented with 0.05% (v/v) Tween. After extensive rinses in sterile distilled water, seedlings were transferred to solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.8% agar (w/v) without growth regulators (pH 5.7). Plants were grown in a climate chamber at 24±2 °C under 16 h light/8 h dark photoperiod and artificial fluorescent lamp (90 μmol s^{-1} m^{-2}) and multiplied by subcultivation every 4 to 6 weeks using nodal segments.

Four-week-old plants were exposed to salt stress by supplementing MS medium with 100 or 200 mM NaCl. MS medium without added NaCl was used as control. After 33 days of treatment, roots were gently separated from shoots and carefully washed to remove medium residues between root branches. Root tissue was stored at –80 °C until analyses. Due to severe growth retardation in plants exposed to the higher concentration of NaCl (200 mM), roots from several plant samples had to be pooled together. Line 244B did not develop a root system at any salt treatment hence this line was omitted from further analyses.

Proline content

Free proline content was determined according to Bates et al. (1973). 10–25 mg of fresh plant tissue was homogenized in 1.5 ml of 3% (w/v) sulphosalicylic acid and the residue was removed by centrifugation at 15 000 g for 15 min. Supernatant, ninhydrin and glacial acetic acid were heated at 100 °C for 1 h, and the reaction was stopped in an ice bath. The absorbance of the free proline fraction with toluene aspirated from the liquid phase was read spectrophotometrically at 520 nm. Proline concentration was determined using a calibration curve obtained with L-proline solutions ranging from 10 to 160 μM and expressed as micromols per gram of fresh weight [μmol $g^{-1}{}_{FW}$].

Enzyme extraction

Root tissue (50 mg) was homogenized in 1 ml ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ascorbic acid and polyvinylpolypyrrolidone (PVPP). Homogenate was centrifuged at 25 000 g for 30 min at 4 °C. The content of soluble proteins in the supernatant was determined according to Bradford (1976) using bovine serum albumin as a standard. Obtained supernatants were used for assays of antioxidative enzyme activities.

Assays of antioxidant enzyme activities

POD (EC 1.11.1.7) activity was analysed by measuring the oxidation of guaiacol in the presence of hydrogen peroxide at 470 nm according to Chance and Maehly (1955). CAT (EC 1.11.1.6) activity was estimated according to Aebi (1984) by monitoring the decline in absorbance as a consequence of hydrogen peroxide consumption at 240 nm. The activity of SOD (EC 1.15.1.1) was determined by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm as described by Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of NBT reduction. The enzyme activities were expressed as units (U) of enzyme activity per milligram of protein [μmol min^{-1} $mg^{-1}{}_{proteins}$].

Statistical analysis

All results were expressed as means of three replicates with the corresponding standard errors, except for lines 238C and 239K at the highest salt concentration (200 mM NaCl) where only one replicate was obtained. Data were subjected to the analysis of variance (ANOVA) with a 3×4 factorial approach replicated three times with missing values. Differences between means were compared at $P \leq 0.05$ of significance using Fisher's least significant difference test.

Principal component analysis (PCA) was performed to evaluate and discriminate the roots responses of different tobacco lines exposed to NaCl. The data set used for PCA comprised 5 variables (protein concentration, proline content, activity of POD, CAT and SOD).

Data were analysed with STATISTICA 10.0 (Stat Soft Inc., USA) software package.

Results

After 33 days of treatment all control plants of tested lines (207B, 239K and 238C) and hybrid DH10 as well as those treated with 100 mM NaCl developed roots. In all tobacco lines roots of control plants were fibrous and thick, 3.5–5 cm long, with lateral branches, while plants exposed to 100 mM NaCl had shorter roots (1–2.5 cm). The most pronounced effect of salt stress was noticed at 200 mM NaCl where growth of roots was severely inhibited and roots were only 0.5–1 cm long (Fig. 1) or even not developed, as in line 244B which was therefore omitted from further analysis.

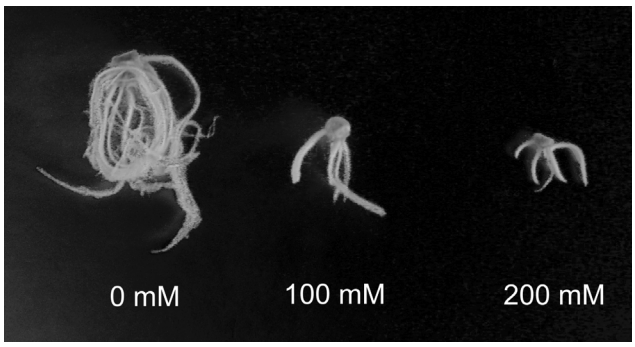

Fig. 1. Roots of line 207B after 33 days of treatment with 0, 100 and 200 mM NaCl.

Considering total protein concentration, results revealed that in line 207B protein concentration was higher in roots of plants exposed to 200 mM NaCl, especially compared to untreated (control) plants (30% above the control value in 207B). Among different tobacco lines control plants of line 239K had the lowest protein content (Fig. 2a).

Salt stress caused remarkable proline accumulation in the roots of all tobacco lines treated with 200 mM NaCl in comparison to their respective controls (from 2-fold above the control level in hybrid DH10 to above 8-fold in 207B). The proline accumulation was lowest in roots of 239K line. Treatment with 100 mM NaCl caused significant proline accumulation in all dihaploid lines (5-fold in 207B and 4-fold in 239K and 238C) but not in hybrid DH10 (Fig. 2b). When the proline content in the control plants of all experimental groups was compared, a significantly higher level was measured in hybrid DH10.

Salt stress mostly did not cause any considerable differences in activities of antioxidative enzymes in the roots of all the tobacco lines tested. However, statistical analysis revealed a higher CAT activity in roots of line 239K exposed to 100 mM NaCl than in the roots of line 207B exposed to the same salt concentration (Fig. 3a). In a comparison of POD activity in control plants of different tobacco lines, line 239K had significantly higher POD activity than line

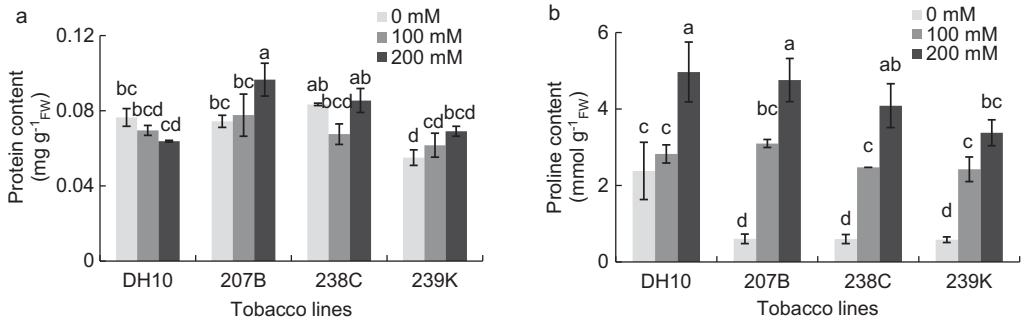

Fig. 2. Protein concentration (a) and proline content (b) in roots of tobacco F1 hybrid DH10 and dihaploid lines 207B, 238C and 239K exposed to 0, 100 and 200 mM NaCl for 33 days. Values are means±SE (n=3). Different letters indicate significantly different values ($P<0.05$) between means.

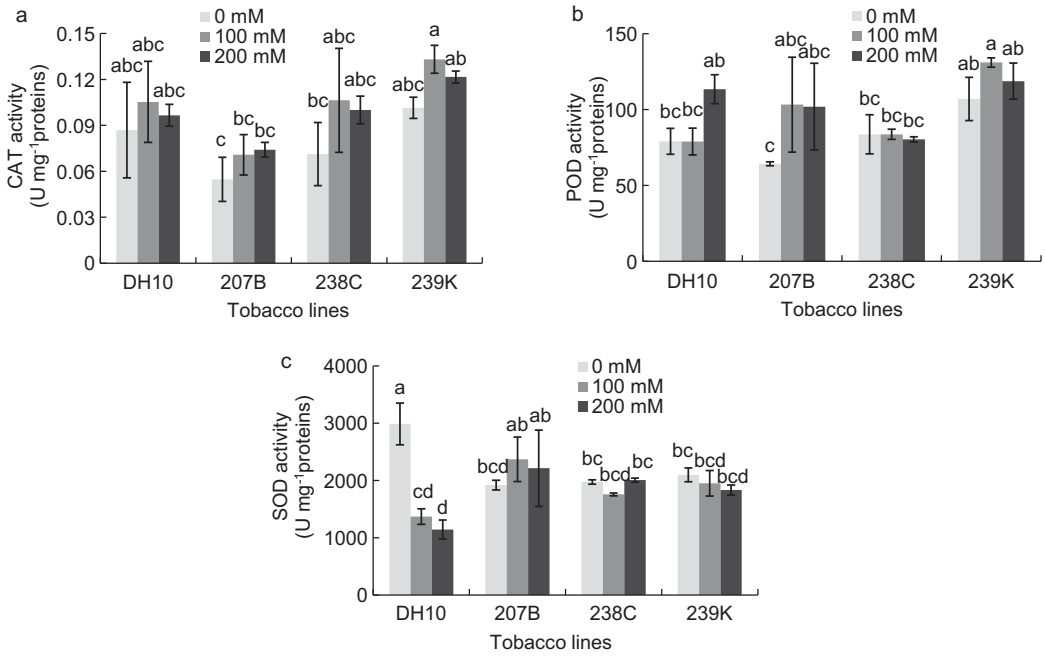

Fig. 3. Activity of catalase (a), peroxidase (b) and superoxide dismutase (c) in roots of tobacco F1 hybrid DH10 and dihaploid lines 207B, 238C and 239K exposed to 0, 100 and 200 mM NaCl for 33 days. Values are means±SE (n=3). Different letters indicate significantly different values ($P<0.05$) between means.

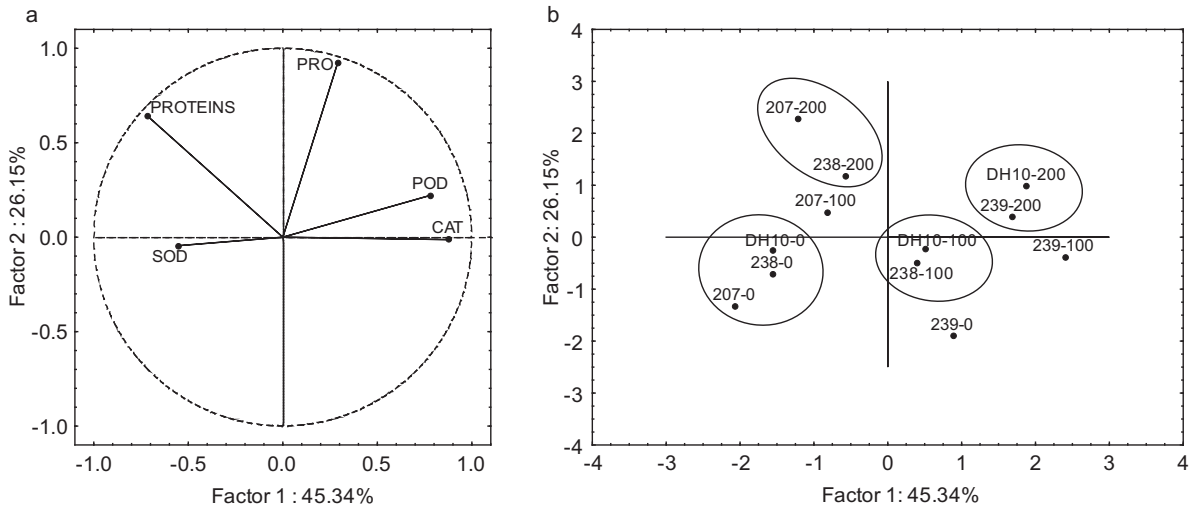

Fig. 4. Principal component analysis of combined tobacco lines data sets. Loadings (a) and scores (b) of the first two factors. Protein concentration (PROTEINS), proline content (PRO) and activity of SOD, POD and CAT represent variables. Numbers 0, 100 and 200 following names of tobacco lines (DH10, 207B, 238C and 239K) represent concentrations of NaCl – 0, 100 and 200 mM, respectively.

207B and increased enzyme activity at 100 mM NaCl in comparison to 238C and DH10 (Fig. 3b). The activity of SOD significantly decreased in hybrid DH10 in NaCl supplemented medium as compared to its respective control. Among different tobacco lines the highest SOD activity was observed in control plants of hybrid DH10 while salt-treated roots of line 207B had significantly higher SOD activity than hybrid DH10 under the same treatment (Fig. 3c).

PCA yielded two significant components explaining 70% of data variance. The first component (PC1) was largely determined by POD and CAT activity as well as protein content and SOD activity and the second component (PC2) by protein and proline contents (Fig. 4a). The corresponding scores plot (Fig. 4b) showed that unstressed plants of lines 207B, 238C and hybrid DH10 grouped together in cluster I while unstressed plants of line 239K separated due to lower protein content. Due to higher CAT activity and higher SOD activity, plants of lines 239K and 207B separated from other lines treated with 100 mM NaCl (DH10 and 238C, that form cluster II). Plants of all lines exposed to 200 mM NaCl were differentiated from others due to their high proline content but they further separated into two clusters, cluster III with plants from lines 207B and 238C characterized with higher protein content, and cluster IV with plants from lines 239K and hybrid DH10 characterized with increased POD activity.

Discussion

Root growth reduction is one of the most common effects of increased salinity (Ceccoli et al. 2011). In our experiment, salt stress caused prominent growth inhibition of the roots of all tobacco lines and hybrid DH10. It even completely inhibited root development, especially in line 244B which was therefore omitted from further analysis. Low water potential of nutrient medium, osmotic and toxic effect of NaCl and disturbed uptake of essential minerals can all account for the observed effect (Haq et al. 2009). Previously obtained results revealed that in tobacco shoots salt stress also reduced growth but not very severely, especially in lines 207B and 239K (Marček et al. 2014). This might indicate that shoots at least partially avoided toxic effect of NaCl, retaining it by apoplastic barriers in root or by disposal into parenchyma cells of the root cortex (Munns and Tester 2008). The same effect was observed in wheat (*Triticum* sp.) (Datta et al. 2009) and Persian clover (*Trifolium resupinatum* L.) (Ates and Tekeli 2007).

With the application of higher NaCl concentration (200 mM) a significant increase in root protein content in line 207B was observed. This effect was determined earlier in roots of wheat cultivars (Shaddad et al. 2013). Increased protein content can be an indicator of salinity tolerance if it is caused by synthesis of stress proteins or proteins contributing to antioxidative defence (Mohammadkhani and Heidari 2008, Manaa et al. 2011). However, a degradation process caused by growth retardation can also increase protein level (Parvaiz and Satyavati 2008, Goudarzi and Pakniyat 2009, Keshtehgar et al. 2013, Gupta and Huang 2014). Contrary to the results obtained in this study, protein content in shoots, investigated previously, remained unchanged within lines 207B and 238C exposed to salinity (Marček et al. 2014). It has been reported that salinity can cause different changes in the protein content in various parts of the plant (Elsamad and Shaddad 1997).

In all dihaploid lines and hybrid DH10, salt stress caused significant increase in proline production in roots, implying that proline contributes to osmotic adjustment. Furthermore, remarkable proline production in 207B, 238C and 239K at both salt treatments suggests that in these genotypes proline could be connected with salt resistance (Ahmed et al. 2008). Interestingly, despite higher basal proline content in control plants, roots of hybrid DH10 did not show any more prominent tolerance to 100 mM NaCl. According to Tammam (2003), sodium accumulation in root causes increased proline production thus protecting root cells against dehydration. Increased proline content was noticed in the root of a

tobacco cultivar (*N. tabacum* L. Wisconsin) exposed to 300 mM NaCl (Razavizadeh et al. 2009). When proline production in shoots of tobacco dihaploid lines and hybrid DH10 exposed to NaCl was considered, significant proline production in plants at 200 mM NaCl was recorded (Marček et al. 2014). Much higher proline content in shoots than in the roots of these lines suggests that tobacco, like most glycophytes, could have a poor ability to exclude salt, so Na^+ is concentrated in the cell vacuoles of transpiring leaves. In such conditions, proline should accumulate in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole (Munns 2002). Similar results have already been reported for barley (*Hordeum vulgare* L.) (Ueda et al. 2007) and maize (*Zea mays* L.) (Versules and Sharp 1999).

CAT, POD and SOD are considered major scavenging enzymes involved in removal of ROS produced under various stress conditions, including salinity (Parvanova et al. 2004). Among investigated tobacco lines, roots of line 239K treated with 100 mM NaCl exhibited higher CAT activity than line 207B and increased POD activity compared to line 238C and hybrid DH10; those increased activities of both enzymes in line 239K might ensure better scavenging of ROS under 100 mM NaCl. On the other hand, roots of lines 207B and 238C treated with 200 mM NaCl showed remarkable induction of SOD activity compared to hybrid DH10 which implies that in the roots of these tobacco lines SOD might be more involved in defence response. In our previous study (Marček et al. 2014), shoots of line 239K also had pronounced SOD, CAT and POD activity under salt levels so it seems that line 239K generally had more effective antioxidative defence mechanism and is thus less susceptible to salt stress than other dihaploids and hybrid DH10. Untreated roots of hybrid DH10 had higher SOD activity than other tobacco lines indicating that its expression is constitutive under physiological level (Rangani et al. 2016). Moreover, roots of hybrid DH10 exposed to salt treatments showed the same inhibition of SOD activity as shoots (Marček et al. 2014) which could be connected with the lack of zinc (Tavallali et al. 2010), an enzyme cofactor, since salinity disturbs plant nutrient uptake and ion distribution (Fernández-García et al. 2004, Bameri et al. 2012). The decline in SOD activity in the salt-exposed roots was also demonstrated in maize salt-sensitive cultivar (SK) (Keyster et al. 2013). Observed differences in enzyme activity between lines included in this experiment might be the result of their genetic properties. Compared to shoots (Marček et al. 2014), the roots of all dihaploids and hybrid DH10 exhibited high CAT, POD and SOD activity as a response to increased salinity suggesting that roots of tobacco dihaploid lines have a stronger antioxidative defence. Similarly, more prominent antioxidative response of root than of shoot has been reported for the lentil (*Lens culinaris* M.) (Bandeoğlu et al. 2004).

In conclusion, NaCl stress caused root growth inhibition and remarkable proline accumulation in all tobacco dihaploid lines 207B, 238C, 239K and hybrid DH10 but certain differences among antioxidative enzyme activities as response to salinity were noticed. Line 239K which expresses higher CAT, POD and SOD activity in shoots of NaCl treated plants (Marček et al. 2014) also showed increased activities of CAT and POD in roots, when compared to some other lines. This suggests that stress responses induced in the root contribute to the salt tolerance of this tobacco line. The variances in antioxidative response imply genetic variability of tobacco lines, which could account for differences in salinity tolerance.

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