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EFFECTS OF MIXED SALINE AND ALKALINE STRESS ON THE MORPHOLOGY AND ANATOMY OF *PISUM SATIVUM* L.: THE ROLE OF PEROXIDASE AND ASCORBATE OXIDASE IN GROWTH REGULATION

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Abstract - The effects of hyperalkaline, thermo-mineral water from Slatina on the morphology and anatomy of pea plants (*Pisum sativum* L.), were examined after eleven days of treatment with a mixture of tap water and Slatina water in 3:1 (T1) and 1:1 ratios (T2). Complete growth arrest of seedlings was observed in the Slatina water (T3). The alkalinity of external media was recovered to pH 8 within four days only in T1 and T2. Analysis of morphological parameters (the length of the main root, root application zone, number of lateral roots) indicated that the thermo-mineral water either promoted (T1) or inhibited (T2) the formation of lateral roots and plant growth. Comparative histological and anatomical analyses showed that inhibition of lateral roots was accompanied by an increase in the xylem and phloem. These changes in root morphology were accompanied by an increase in the activity of superoxide dismutase (SOD: E.C. 1.15.1.1) and peroxidase (POD: E.C 1.1.1.17) in the soluble fraction, whereas the activities of ascorbate oxidase (AAO: E.C. 1.10.3.3) bound to the cell wall and ionic POD decreased. The lower ratio of Slatina water in the hydroponic solution contributed to a more developed mesophyll with significantly higher AAO activity in the leaves and the induction of ionic POD isoforms. Besides alkalinity and excess NaCl, we suggest that a specific combination of metals (e.g. Ca and Mg) might be responsible for subtle changes in the cell area and xylem development, leading to dramatic changes in root anatomy.

Key words: Salinity, alkalinity, pea, hyperalkaline water, peroxidases, ascorbate oxidases, growth

Abbreviations: abscisic acid – ABA; ascorbate oxidase – AAO; electrical conductivity – EC; chlorophyll a – Chla; chlorophyll b – Chlb; reactive oxygen species – ROS; peroxidase –POD; phenylmethylsulfonyl fluoride – PMSF; superoxide dismutase -SOD.

INTRODUCTION

Salinization and alkalization are dynamic soil degradation processes affecting more than 10% of world arable land and limiting agricultural production (Läuchli and Lüttge, 2002). Saline soils with electrical conductivity (EC) >4 dS/m and high concentrations of neutral salts (NaCl, Na_2SO_4) impose osmotic stress and ion-induced injury in plants (Munns, 2002; Shi and Sheng, 2005; Shi and Wang, 2005; Zhang and Mu, 2009). Salt-tolerant plants, halophytes, can survive salinity in excess of 300 mmol/L, while glycophytes are inhibited at 100-200 mmol/L (Zhu, 2007). Alkaline stress caused by high concen-

trations of alkaline salts ($NaHCO_3$ and Na_2CO_3) has an additional effect on plants due to high pH, and is more severe than salt stress (El-Samad and Shaddad, 1996; Campbell and Nishio, 2000; Hartung et al., 2002; Shi and Sheng, 2005; Shi and Wang, 2005; Rao et al., 2008, Yang et al., 2007, 2008).

A highly alkaline environment (pH >8.5) can induce growth and photosynthesis inhibition, alterations in antioxidative metabolism, ion accumulation, destruction of the structure of root cells and even leads to cell death (Tang and Turner, 1999; Hartung et al., 2002; Zhang and Mu, 2009; Li et al., 2010). Relatively little attention has been given to the effect of both salt and alkaline stress on plants, as such conditions frequently co-occur, especially from the aspect of perturbation of redox homeostasis, reactive oxygen species (ROS) levels and antioxidative enzymes which regulate growth through cross-talk with hormones such as auxin and abscisic acid (ABA) (Foyer and Noctor, 2003; Foyer, 2005). We measured the changes in the activity and abundance of two ROS-scavenging enzymes, superoxide dismutase and peroxidase, the main enzymes controlling the levels of superoxide anion and hydrogen peroxide in plants. In addition to their H_2O_2-scavenging function, a number of POD isozymes catalyze oxidative cross-linking of aromatic compounds leading to cell-wall stiffening (Quiroga et al., 2000) and formation of a secondary cell wall through lignification (Lewis and Yamamoto, 1990; Polle et al., 1994) and suberization (Bernards et al., 1999). However, POD can contribute to the production of $\cdot OH$ radicals and cause cell wall loosening as well (Fry, 1986; Chen ad Schopffer, 1999; Kukavica et al., 2009). In addition, POD is involved in auxin oxidation (Gazaryan et al., 1996) and plant defense mechanisms (McLusky et al., 1999). Most of the functions of cell wall-bound POD isoforms take place in the apoplast, thereby presenting an important regulatory factor of cell elongation and growth. Besides H_2O_2 and phenolic compounds in the apoplast, the ascorbate level is an additional regulatory growth factor (Diallinas et al., 1997). Ascorbate oxidase is a Cu-containing enzyme that oxidizes ascorbic acid to dehydroascorbic acid, accompanied by the reduction of molecular oxygen to water (Esaka et al., 1992). The physiological role of AAO is still unclear; however, its cell wall localization and high activity in rapidly expanding tissues indicate its involvement in cell elongation (Esaka et al., 1992; Moser and Kanellis, 1994; Ohkawa et al., 1994; Kato and Esaka, 1999).

In this study, we used hyperalkaline water from the water spring Slatina, which is located 16 km northeast of Novi Grad, the Republic of Srpska (Bosnia and Herzegovina). While this water is well known for its medicinal use, there is no information on its effect on soil and plants. According to its physicochemical properties, the Slatina water belongs to a thermo-mineral sodium chloride-type of water, with a pH value ranging between 11.85 and 12.10 and an EC of 5.12 dS/m (Table 1). In order to investigate the effects of Slatina water on plant development and the ability of plants to regulate extremely high media pH, *Pisum sativum* L. seedlings were treated with different ratios of tap and Slatina water under controlled conditions. The observed changes in development, activities of SOD, POD and AAO and redox status that were induced by the specific mineral composition and high pH of the Slatina water are discussed in relation to growth regulation.

MATERIALS AND METHODS:

Experimental conditions

According to its physico-chemical analysis, water from Slatina can be classified as a sodium chloride-type of water with a very high pH. Compared to tap water, it contains several-fold higher concentrations of Na^+ and Cl^- ions and an elevated concentration of K^+ ions (Table 1). Water collected from the Slatina spring was immediately used for hydroponic experiments. The seeds of pea plants (*Pisum sativum* L.) were germinated on moist filter paper. After three days, the seedlings were transferred to pots with different ratios of tap water/Slatina water: T1 – tap water/Slatina 3:1; T2 – tap water/Slatina 1:1; and control plants grown only in tap water. The plants were harvested and used for morphological, anatomical

and biochemical analyses 11 days after the beginning of the treatment. The experiment was performed under controlled conditions at 60% humidity, 25˚C, a photoperiod of 16 h of light and irradiance with 250 μmol photons m^{-2} s^{-1}.

Biomass determination and analysis of the mineral composition

The leaves and roots of 11-day-old plants were harvested and the fresh biomass was measured. Samples were dried in an oven at 60°C for 48 h and the dry biomass measured. For determination of the mineral content, dried samples of leaves and roots were milled and digested in 3 ml 69% HNO_3 and 2 ml 30% H_2O_2 using a microwave digestion system (Mod. speedwave MWS3+, Berghof, Germany). Water samples from the Slatina spring and tap water were acidified with HNO_3 to pH <2. The mineral content was determined by ICP-AES (Spectro Genesis FEE, Kleve, Germany). Ten plants were randomly selected for each treatment and the control. The following morphological parameters were measured: stem length, the length of the main root, the number and lengths of the lateral roots and the root application zone. The results were statistically processed using Maple (standard *t* test on 2 samples with equal variance).

Morphological and anatomical analysis

For comparative anatomical/histological analysis, 10 plants were randomly selected in every treatment. A cross section was made in the middle of the first right lamina of the complex leaf and stomata type, determined according to the classification of Metcalfe (1957). Roots were analyzed in three zones (Fig. 5). The first section (Zone 1) was below the lateral root formation (the middle of the elongation zone); the second (Zone 2) was in the middle of the lateral root formation zone, and the third (Zone 3) was 5 mm above Zone 2 (Fig. 5). Samples were cut to a thickness of 20 μm with a microtome. The sections were fixed and stained, first with safranin and alcian blue, then with hematoxylin (Chamberlain, 1921). Fingerprints of perianth tissue were taken according to Wolf (1973). Microscopic lamina thickness, cell height and the adaxial and abaxial epidermis areas, palisade and spongy tissue in leaves, and the cell area in all three intersection zones, as well as the area of xylem and phloem tubes in the roots were analyzed using an Olympus microscope VANOX AH 2 at a magnification of 10x40 (Figs. 4,5). Images were taken with an Olympus DP 12 camera. The results were analyzed with a software package IMAGE ANALYSER Olympus. The results were statistically analyzed using statistical the Maple10 package with a limit for the level of significance of 95%.

Protein extraction and enzyme analysis

To obtain soluble proteins from leaves and roots of the 11-day-old pea plants, samples were homogenized in 0.1 M sodium-phosphate buffer, pH 6.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4,000 x g for 15 min at 4°C. After centrifugation, the supernatant containing soluble proteins was separated from the pellet that was re-extracted with 0.1 M sodium-phosphate buffer, pH 6.4, containing 1 M NaCl, on ice for 30 min with occasional mixing. The homogenate was centrifuged at 4,000 x g for 15 min at 4°C in order to separate the ionic cell wall fraction in the supernatant. The protein content was determined according to Lowry (1951).

Covalent cell wall fractions were isolated from the pea roots following the method described by Kukavica et al. (2009) with minor modifications. The roots were powdered in liquid N_2 and homogenized in 50 mM Tris/HCl (pH 7.2) buffer containing 50 mM NaCl, 0.05% Tween 80, 1 mM PMSF. The homogenate was filtered through two layers of cloth and centrifuged at 1,000 x g for 15 min at 4°C. The cell wall pellet was washed four times in the same buffer without the detergent and salt and then suspended in 10 ml of 50 mM Tris/HCl (pH 7.2) buffer containing 1 M NaCl, followed by incubation for 30 min at 4°C and centrifugation at 1,000 x g for 15 min. The cell wall pellets were washed with 50 mM Tris/HCl (pH 7.2) several times, and then used for the the

determination of AAO activity according to Morina et al. (2010).

POD activity was determined in the soluble and ionic cell-wall bound fractions of pea leaves and roots. Pyrogallol (A_{430}; $\varepsilon_{purpurogallin} = 12\ mM^{-1}\ cm^{-1}$) was used as a hydrogen donor. Absorbance was measured at 430 nm. The reaction mixture consisted of 20 mM pyrogallol, 3.3 mM H_2O_2 in 100 mM sodium-phosphate buffer (pH 6.4) and an aliquot of the extract. POD isoforms were separated by native electrophoresis on a 10% polyacrylamide gel at 100 V for 120 min. For the detection of POD isoforms, the gel was incubated in 0.01% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM potassium-phosphate buffer (pH 6.5).

SOD isoforms were separated by PAGE and detected according to Beauchamp and Fridovich (1971) after incubating the gel in a reaction mixture containing 0.01 M EDTA, 0.098 mM nitroblue tetrazolium, 0.030 mM riboflavin and 2 mM TEMED in 50 mM potassium phosphate (pH 7.8) for 30 min in the dark, followed by washing with distilled water and illumination with a fluorescent lamp (30 μEm^2s^{-1}) for 15 min. Total SOD activity and the activity of individual SOD isoforms from the native gel were calculated using TotaLab software.

Determination of photosynthetic pigments

Chlorophyll a and b and carotenoids were extracted in 100% acetone (0.5 g_{FW} plant material/5 ml acetone). After centrifugation at 3,000 rpm for 15 min, the supernatant was used and absorbance measured. The concentrations of chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoids were calculated according to Holm (1954) and Van Wattstein (1957).

Statistical analysis

The data were analyzed with the Statistica 6 program for Windows. The significance of differences between control and treated plants was determined by Student's t test. The significance threshold was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Morphological changes during plant growth

Pea seedlings were grown hydroponically for eleven days in a mixture of tap and Slatina water as described, while the plants grown only in tap water served as a control (Fig. 1). Under these conditions, the pea plants were exposed to three different levels of combined salt stress caused by neutral salts, and alkaline stress. The growth of plants in pure Slatina water was completely inhibited, and these plants were not used for further analysis. While no significant changes in the dry biomass were obtained in the T1 treatment, significant growth inhibition was observed in the leaves and roots of the T2 plants. The fresh weight of T1 leaves was higher than in the control plants (Table 2). Morphological analysis indicated that the specific mineral composition and alkalinity of the Slatina water affected root and leaf development. The T1 treatment had a stimulatory effect on elongation of the main root (which was 22% longer than in the control), and on the formation of new lateral roots (Fig. 3). On the other hand, a higher percentage of Slatina water in the hydroponic medium was inhibitory for root branching, observed as a significantly reduced root application zone (3.5 times) and a lower number of lateral roots (up to 78%). Surprisingly, the T2 plants developed and a few had the longest lateral roots (Fig. 3). The multiple effects of mixed saline-alkaline stress on plants was shown earlier (Shi and Wang, 2005; Yang et al., 2007; Zhang and Mu, 2009). In general, salinity and alkalinity can reduce leaf number and area, shoot and root dry weight, thus causing lower plant yields (Mohamedin et al., 2004; Essa, 2002; Li et al., 2006; Sharifi et al., 2007). However, plant growth can be stimulated at low salinity (Marschner, 1995; Koyro, 2006), which could explain our results on the stimulation of elongation and branching of the main root of the T1-treated plants.

Changes in anatomy of leaves and roots

Pea leaves are amphistomatic with a poorly devel-

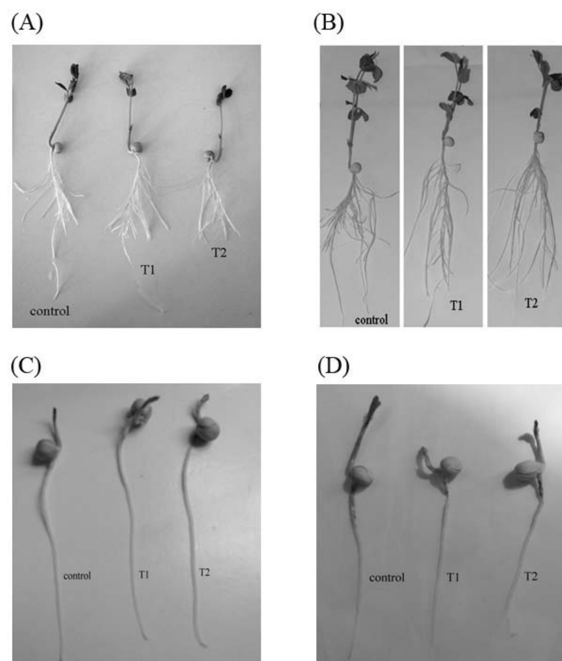

Fig. 1. Pea plants grown in tap water (control) and in a mixture of tap water and Slatina thermo-mineral water (in treatment T1, the ratio of tap water to Slatina water was 1:3; in T2 it was 1:1). (A) 5-day-old and (B) 11-day-old plants. Pea plants grown in pure Slatina water: (C) 5-day-old plants and (D) 11-day-old plants.

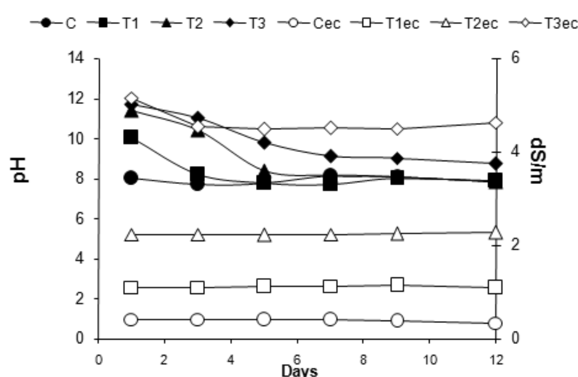

Fig. 2. Changes in pH and electrical conductivity (EC, dS/m) of the growing media during 11 days of the experiment. Control-tap water; T1 - tap water : Slatina water = 3:1; T2 - tap water : Slatina water = 1:1; pure Slatina water. The data are presented as the means ± STDEV. *p≤0.05.

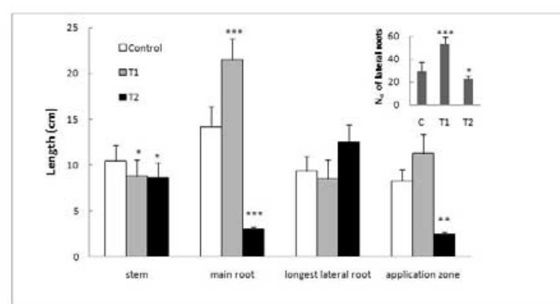

Fig. 3. Morphological analysis of control (C) and treated pea plants (T1 and T2); stem length, main root length, root application zone, the largest lateral root and number of lateral roots are presented. The data are presented as the means ± STDEV. * p≤0.05, ** p≤0.01, *** p≤0.001.

oped mesophyll consisting of one layer of palisade and five to six layers of spongy tissue (data not shown). T1 and T2 treatments affected the size of the palisade and epidermal cells only on the adaxial side. T1 plants had the thickest leaves with a better-developed mesophyll, height and area of palisade and spongy cells compared to the control. This led us to assume that the process of photosynthesis in these plants was more intensive (Fig. 4; Table 5). However, a decrease in both cell height and cell area of palisade and spongy tissues in the treated plants was observed in the T2 plants (Table 5). Similar changes in the leaf anatomy, such as the number and size of the stomata, the decrease of the diameter and number of xylem vessels and the inhibition of tissue differentiation due to salt excess, have already been observed for other species (Waisel, 1972; Solomon et al., 1986; Curtis and Lauchli, 1987; Dolatabadian et al., 2011).

Pea roots are spindle-shaped with pronounced lateral roots. Radial vascular tissue in the central cylinder consists of three phloem and xylem tubes. The average value of the cell area in Zone 1 (elongation zone) increased in T1 and T2 plants compared to the controls (Fig. 5, Table 6), while it decreased in both treatments in Zone 3 (differentiated zone). According to West et al. (2004), salt stress inhibits cell division and the cell cycle, as well as cell elongation, which can explain the changes in the cell area observed in our experiment. The roots of T2-treated plants had better developed conductive tissues (xylem along all three zones and phloem in the zone of lateral roots) and larger cell area (Table 6). In addition, these roots were the shortest due to growth in-

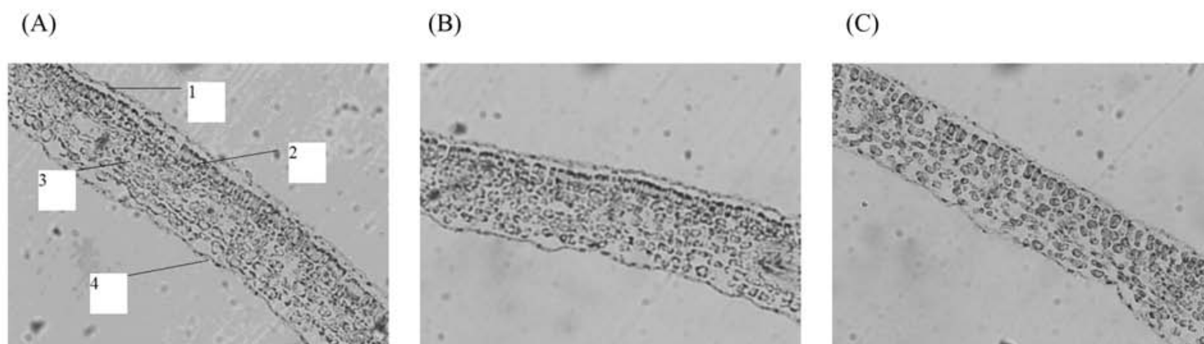

Fig. 4. Leaf cross-section: A) control: numbers indicate different leaf tissue: 1 - adaxial epidermis; 2 - palisade tissue; 3 - spongy tissue; 4 - abaxial epidermis. B) T1 plants. C) T2 plants.

hibition, which may have caused the observed root thickening. Roots of the T1 plants had the longest main root and the largest number of lateral roots, although the cell area and conductive tissues were less developed compared to the T2 plants. Root-growth inhibition and thickening and the overall reduced development of the T2 plants were caused by the unfavorable conditions of mixed alkaline/saline water. According to our results, the T1 plants had the most progressive development, due to the larger palisade tissue area in the leaves and phloem area in Zone 3 (Tables 5, 6).

Perturbations of pH, EC and elements in the growing medium and in plants

During the experiment, the pH and EC of the hydroponic solutions were measured every day for every treatment. At the beginning of the experiment, the pH was 10.4, 11.4, and 11.9 in T1, T2 and the pure Slatina water-treated groups, respectively. After four days, the pH of the hydroponic solutions in T1 and T2 decreased to almost neutral and was not significantly different compared to the control (pH=8) (Fig. 2). Pea seedlings grown in pure Slatina water, which exhibited retarded growth from the very beginning, were not capable of recovering the pH of the external media. The conductivity of the different growing media at the beginning of the experiment (5.12 dS/m for pure Slatina water; 1.53 dS/m for T1; and 2.42 dS/m for T2) were higher compared to tap water (0.427 dS/m) (Table 1). In contrast to the pH changes during the experiment, the EC of the hydroponic solutions did not change significantly in any of the treatments (Fig. 2). It decreased to 83% and 88% after 11 days in the control and T2, respectively, while in T1 it did not change.

Changes in the composition of elements in the leaves and roots of pea plants were observed in the T1 and T2 groups (Table 3). The significant increase in Na^+ content was obtained in both leaves and roots: 8.6 and 15 times higher compared to the leaves of control plants, and 2.3 and 3.5 times higher compared to the roots of control plants for the T1 and T2 treatments, respectively. Compared to the control, the concentration of Ca^{2+} in the leaf and root decreased in both treatments. In addition, a significant reduction in Mg^{2+} concentration was observed only in the roots of T1- and T2-treated plants, while the concentration of K^+ slightly increased in the roots of the T2 plants. The accumulation of Na^+ in the plant tissue can be accompanied by a decrease in Ca^{2+} and Mg^{2+} concentrations due to the decreased influx and reduced xylem loading and salt-induced transcriptional regulation of Ca^{2+} and Mg^{2+} transporters (Cramer et al., 1986; Cheeseman, 1988; Parida and Das, 2005; Maathuis 2006). In order to overcome the initial osmotic stress induced by the more negative water potential of the surrounding environment, the uptake of inorganic ions is stimulated (Marschner, 1995). Na^+ enters the plant cells either through a high-affinity K^+ transporter or via non-selective cation channels (Zhu, 2003). Besides, salt stress may

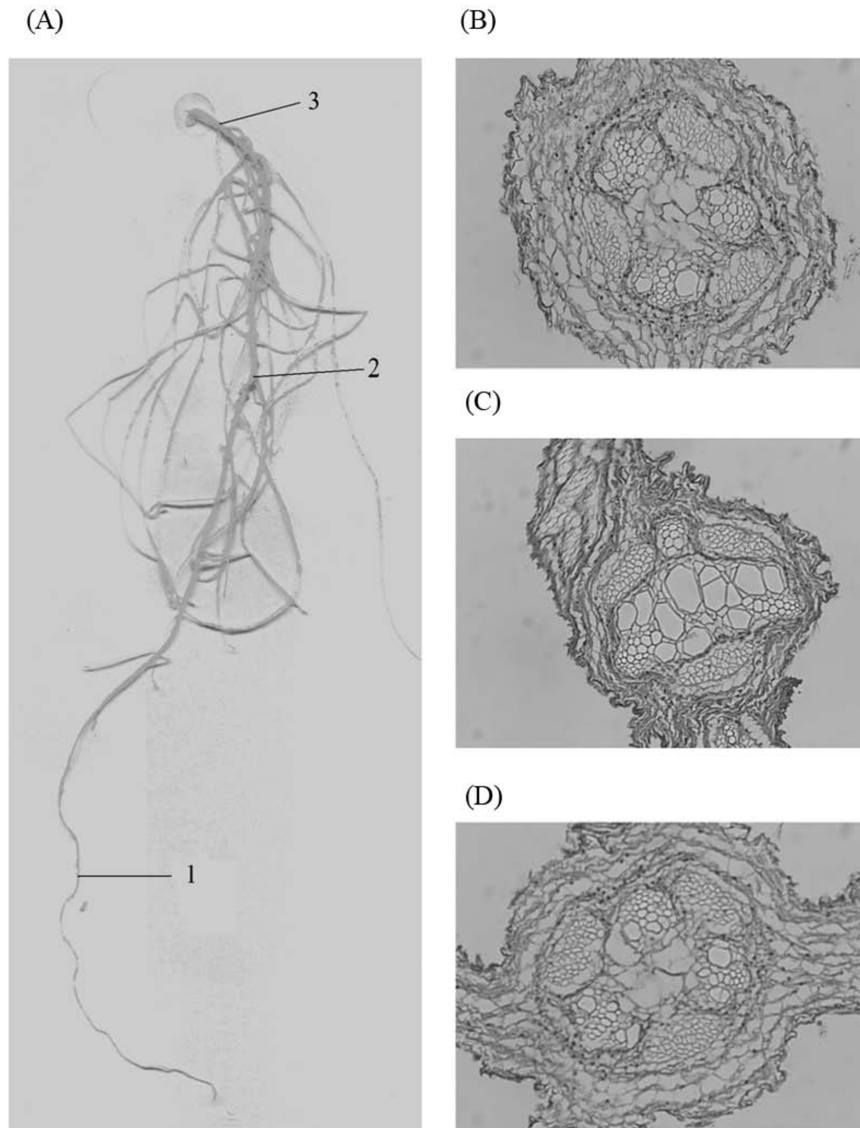

Fig. 5. A) Pea root: numbers indicate 3 zones where the cross-sections were made: Zone 1 – the middle part of the root before the formation of lateral roots-elongation zone; Zone 2 – in the middle of the section with lateral roots; Zone 3 – 5 mm above zone 2. Intersection in zone 3: B) control; C) T1; D) T2.

induce the activity of plasmalemma H^+-ATPase (Wu et al. 1998, Marschner 1995). An intensive excretion of H^+ into the external media during the first days of the experiment (Fig. 2) might be coupled to Na^+ import, leading to the accumulation in the vacuole for osmotic adjustment (Table 3). Vacuolar Na^+ sequestration is regulated by K^+ and Na^+/H^+ antiporters that exchange cytoplasmic Na^+ with H^+ ions (Munns and Tester, 2008; Zhu, 2003). We propose that the developmental changes in pea seedlings observed in our work are due to nutrient perturbations, mainly Na^+ accumulation and decrease in the concentrations of Ca^{2+} in the leaf and root, and of Mg^{2+} in the root. Elevated Na^+ concentrations in extracellular media and in cellular compartments influence the acquisition and homeostasis of nutrients. Due to the large

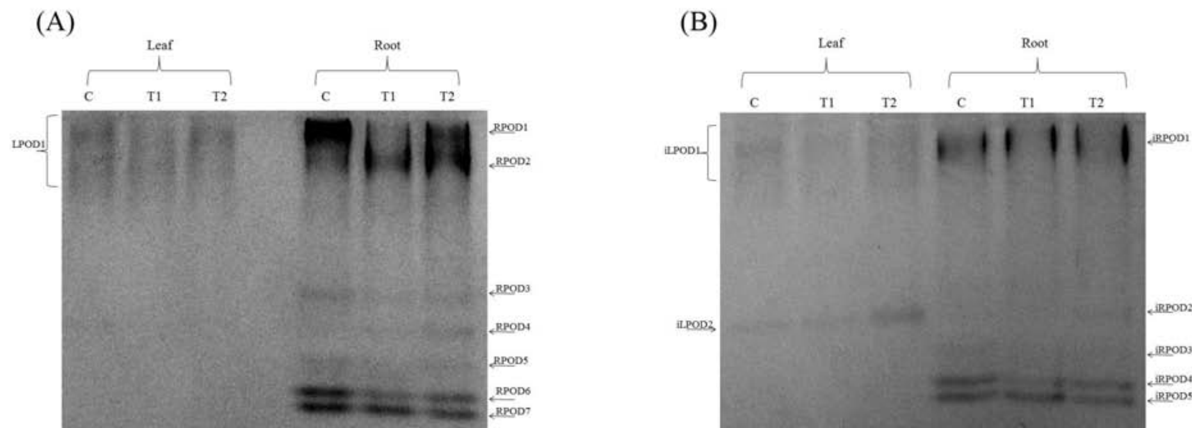

Fig. 6. Separation of A) soluble POD isoforms and B) ionic cell wall-associated POD isoforms by native electrophoresis in leaves and roots after 11 days in control (C) and treated plants (T1, T2). Arrows indicate different POD isoforms.

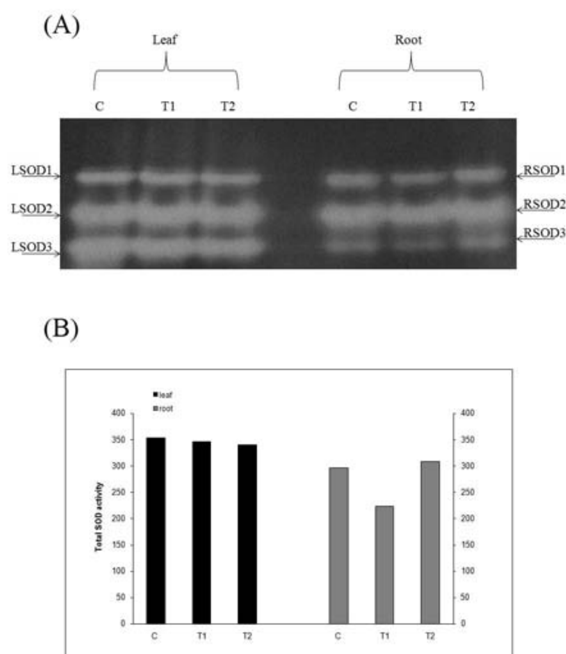

Fig. 7. A) Separation of SOD isoforms by native electrophoresis in leaves and roots after 11 days of growth of control (C) and treated plants (T1, T2). B) Total SOD activity represents the sum of the activities of three individual SOD isoforms detected in leaves and roots of control and treated plants. The activities of individual SOD isoforms were calculated using the TotaLab software.

molar ratio of Na^+/Ca^{2+}, dissociation of Ca^{2+} from its binding sites in the cell wall and plasma membranes occurs, affecting cell wall and plasma membrane integrity. Since Ca^{2+} is relatively immobile, a reduction of transpiration due to osmotic stress may reduce root-shoot Ca^{2+} translocation (Maathuis, 2006).

No significant difference in the content of soluble proteins was obtained in the leaves of treated plants compared to controls. However, the alkaline/salt conditions brought about a progressive decrease in the content of soluble proteins in the roots of T1 and T2 plants (data not shown). Though at high pH values of medium micronutrients (especially iron) become less soluble, resulting in lower uptake and chlorosis (Romheld and Marschner, 1986; Morrissey and Guerinot, 2009), neither chlorosis nor a decrease in Fe^{2+} were detected (Tables 3, 4). High levels of Na^+ may affect chlorophyll biosynthesis. A decrease in Chla and Chlb contents was observed in several plant species exposed to salt and alkaline stress (Agastian et al., 2000; Santos et al., 2004; Radi et al., 2012). The content of Chlb was significantly lower in T1 plants compared to the control, while in T2 plants it was slightly higher but still lower than in the control plants. On the other hand, the content of carotenoids was slightly reduced in both treatments (Table 4).

Induced changes in the activities of antioxidative enzymes

After 11 days of growth in hydroponic culture, we

Table 1. Composition of dissolved elements and electrical conductivity (EC) of water Slatina and tap water (C-control).

Elements (mg/L)	Slatina	Tap water- C
Na^+	940	9.69
K^+	12	0
Al^{3+}	0.6	0.04
B^+	0.5	0
Ca^{2+}	18	38
CO_3^{2-}	42.14	3.8
Cu^{2+}	0	0.001
Cl^-	1067	1.3
Mg^{2+}	0.08	6.7
Mn^{2+}	0	0.01
Mo	2.8	0.03
S	4.9	17
Zn^{2+}	0.3	0.025
EC (dS/m)	5.12	0.427

Table 2. Fresh and dry biomass (g) of shoots and roots of control, T1, T2 and T3 plants after eleven days of treatment. In T3 treatments shoot development was completely inhibited. Data represent means ± STDEV. * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$.

	Shoot		Root	
	Fresh weight	Dry weight	Fresh weight	Dry weight
Control	0.412±0.064	0.045±0.005	0.368±0.071	0.038±0.004
T1	0.488±0.075*	0.047±0.004	0.512±0.062**	0.033±0.003
T2	0.385±0.040	0.033±0.006***	0.454±0.071	0.029±0.004**
T3	-	-	0.012±0.004***	0.001±0.0002***

Table 3. Element composition (mg/kg_{DW}) in pea leaves and roots after eleven days in tap water (control) or on a mixture of tap water and Slatina (T1 and T2). Data represent means ± STDEV. * $p \le 0.05$, ** $p \le 0.01$

	Leaves			Roots		
Element	Control	T1	T2	Control	T1	T2
Na^+	422±79	3650±523**	6602±830**	6537±1007	15035±289**	23001±2100**
K^+	13870±967	14587±1810	15154±2100	11294±714	12957±2390	27095±4300*
Ca^{2+}	8835±781	4379±863**	2248±297**	9572±1200	5430±970**	6309±1150*
Mg^{2+}	1772±508	2036±479	1911±420	12618±1170	4360±703**	2563±557**
S	8013±1356	7490±1200	6348±980	21141±3210	17164±694	14117±2280*
B	23±6	28±7	32±6	17±3	31±6*	69±11*
Al^{3+}	28±1	20±3	25±10	127±32	175±16	133±13
Cu^{2+}	18±3	16±3	18±3	12±2	14±0.1	12±5
Fe^{2+}	94±16	76±12	83±13	60±13	43±15	62±20
Mn^{2+}	16±2	13±2	13±2	4.1±0.2	5.44±0.04	4.98±2
Zn^{2+}	65±14	55±13	62±9	59±14	31±9	81±14

measured the activities and isoform abundance of two antioxidative enzymes, SOD and POD, and the activity of cell wall-bound ascorbate oxidase in both the leaves and roots. Developmental changes in morphology (Fig. 3) and anatomy (Fig. 5, Tables 6) that were provoked by the perturbation in the mineral content of the root tissue and increased external pH may have in part been a result of decreased ionic

Table 4. Content of chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll, ratio Chla/Chlb and content of carotenoids in leaves of control and treated pea plants. Chloroplasts pigments were extracted with 100% acetone and expressed in mg/g_{FW}. Data represent means ± STDEV (* $p \leq 0.05$)

	Chla	Chlb	Chla+b	Chla/Chlb	carotenoids
Control	0.971±0.107	0.788±0.036	1.758±0.086	1.23±0.17	0.196±0.012
Treatment T1	0.944±0.057	0.701±0.029*	1.646±0.087	1.34±0.03	0.184±0.008
Treatment T2	0.982±0.06	0.744±0.038	1.726±0.094	1.31±0.05	0.176±0.014

Table 5. Leaf anatomy: leaf thickness and cell area and height of adaxial and abaxial epidermis, palisade and spongy tissue. Data represent means ± STDEV (* $p \leq 0.05$)

		Control	Treatment T1	Treatment T2
Cell area (μm^2)	Adaxial epidermis	110±8.6	110±6.5	81±8*
	Abaxial epidermis	66±7.6	67±7	46±5*
	Palisade tissue	178±29	185±17	146±10
	Spongy tissue	109±24	117±23.5	99±12
Cell height (μm)	Adaxial epidermis	7.4±1.2	8.7±0.54	7.44±1.90
	Palisade tissue	34±3	37±4	23±10
	Spongy tissue	88±3.6	96±7	94±10
	Abaxial epidermis	4.54±0.44	5±1	4.40±0.31
Leaf thickness (μm)		134±5	148±7*	130±9

Table 6. Root anatomy: root cell area (mm^2) in zone 1, 2 and 3, and xylem and phloem cell area in Zones 1, 2, 3 as indicate on Figure 6. Data represent means ± STDEV (* $p \leq 0.05$, ** $p \leq 0.01$)

		Control	Treatment T1	Treatment T2
Cell area (mm^2)	Zone 1	18±2.5	24±0.2	27±4.6
	Zone 2	73±12.6	51±7	82±10
	Zone 3	568±37	26 ±4**	388. ±69*
Xylem area (mm^2)	Zone 1	3.2±0.42	2.7±0.86	5.6±0.6**
	Zone 2	14.8±2.3	15±7.7	17.5±1
	Zone 3	44±2	47±8.4	53±8
Phloem area (mm^2)	Zone 1	-	-	-
	Zone 2	10±3.5	12±0.6	14±1.4
	Zone 3	16±1	16±1.2	16±2

Table 7. Peroxidase activities expressed as μmol/g_{FW}/min in soluble and ionically bound protein fractions of pea leaves and roots. Data represent means ± STDEV. * $p \leq 0.05$, ** $p \leq 0.01$

	Soluble protein fraction (μmol/g_{FW}/min)		Ionic cell wall fraction (μmol/g_{FW}/min)	
	Leaves	Roots	Leaves	Roots
Control	11.729±0.67	53.78±3-97	3.85±0.439	13.065±1.4
Treatment T1	14.79±1.87	57.64±2.91	2.988±0.236	7.17±0.68**
Treatment T2	16.7±0.89**	19.28±5.79**	1.847±0.139*	8.86±0.766*

Table 8. Ascorbate oxidase activity in the covalent cell wall fraction of leaves and roots. Data represent means ± STDEV. * $p \leq 0.05$, ** $p \leq 0.01$

	($\mu molO_2$/min/OD)	
	Leaves	Roots
Control	155±18	2516±243
Treatment T1	244±29*	1866±148*
Treatment T2	297±25**	1786±208**

POD activity in both treatments, and of soluble POD in T2, and of SOD in T1 (Table 7, Fig. 7). In general, a lower content of spring water in the hydroponic solution had a stimulatory effect on root branching and elongation of the main root. This was accompanied by a lower activity of SOD (Figs. 3, 7). In addition, a remarkable decrease in the specific activity of soluble POD and in the intensity of several bands (RPOD2, 6, 7 and 8) in the roots of the T1 plants confirmed that low salinity improved the redox status of these plants (Fig. 6, Table 7).

The observed changes in the development of pea plants were related to the activity of the cell wall-bound enzyme AAO (Table 8) which is important for apoplastic redox status and growth. While soluble SOD and POD did not change significantly in the leaves, the activity of AAO increased two-fold in the leaves of both T1 and T2 plants (Table 8). Recent findings on the role of AAO in plant systems have shown that the leaves of plants over-expressing AAO had reduced stomatal conductance, higher water content and reduced water loss compared to the wild type (Fotopoulos et al., 2008). This could be of importance for plants exposed to osmotic stress. On the contrary, the growth inhibitory concentrations of elements from the mineral water, and high pH, which resulted in more differentiated conductive cells in the T2 plants, caused an increase in the specific activities of soluble POD and SOD. This suggests that oxidative stress occurred due to the accumulation of ROS induced by salt and alkaline stress. However, the activity of AAO decreased by about 28% in the roots of the treated plants compared to the controls (Table 8). Plants with suppressed expression of AAO proved to be more tolerant to salt stress than the wild type and accumulated less H_2O_2 (Yamamoto et al., 2005). Differences in AAO activity could explain the changes in cell growth and elongation detected in pea roots (Table 8). The system for H_2O_2 scavenging, including ascorbate, POD and phenolics, might become a preferable system for ascorbate utilization in the apoplast compared to oxidation by oxygen.

CONCLUSIONS

In this paper, we showed that mixtures of tap and Slatina thermo-mineral water induced developmental changes in pea seedlings, by combined alkaline and salt stress. Our results show that pea plants grown in hydroponic culture managed to adjust the pH from very high (11.35) to the control levels (7.35) within four days, presumably by the increased activity of the Na^+/H^+ antiporter and/or exclusion of organic and free amino acids (Mane et al., 2010, Yang et al., 2012). Specific morphological and anatomical changes without chlorosis or growth inhibition in the T1 treatment were accompanied by significant changes in the activities of POD and AAO, indicating that the mineral content of the Slatina water has the capacity for developmental regulation. Besides alkalinity and salinity, we suggest that the specific combination of metals (Ca and Mg) might have been responsible for the subtle changes in cell area and xylem development which led to more dramatic changes in root anatomy.

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