NRC Research Press

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Effect of salinity on rhizosphere acidification and antioxidant activity of two acacia species

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Abstract: Salinity is a major environmental stress that is reducing crop yield, particularly in arid and semi-arid regions of the world. It is responsible for many physiological and biochemical disorders in plants. To investigate its effects on two acacia species, namely *Acacia ampliceps* Maslin and *Acacia nilotica* L., a solution-culture experiment was conducted in which both species were exposed to NaCl concentrations of 100 and 200 mmol·L^{-1}. After four weeks of salinity stress, plants were harvested, and physical-growth data were recorded. The membrane stability index and activities of antioxidants enzymes such as superoxide dismutase, peroxidase, and catalase were determined using standard methods. Root exudates were collected for the analysis of organic acids, i.e., citric acid and tartaric acid. Root ash alkalinity was also measured. The results indicated that salinity stress caused a significant increase in the activities of antioxidant enzymes and in the release of organic acids in both species. Higher activities of antioxidant enzymes resulted in less damage to membranes and produced more shoot and root biomass in *A. ampliceps* than in *A. nilotica*. Likewise, having more rhizosphere acidification enabled *A. ampliceps* to respond in a better way to salinity stress than *A. nilotica*.

Key words: salinity, ash alkalinity, citric acid, catalase, peroxidase.

Résumé : La salinité est un stress environnemental majeur qui réduit le rendement des cultures particulièrement dans les régions arides et semi-arides du globe. Elle est responsable de plusieurs désordres physiologiques et biochimiques chez les plantes. Afin d'étudier ses effets sur deux espèces d'acacia, à savoir *Acacia ampliceps* Maslin et *A. nilotica* L., une expérience en milieu de culture liquide où les deux espèces ont été exposées à des concentrations de 100 et 200 mmol·L^{-1} de NaCl a été réalisée. Après quatre semaines de stress salin, les plantes ont été récoltées et des données de croissance ont été notées. L'indice de stabilité membranaire et l'activité d'enzymes antioxydantes telles que la superoxyde dismutase, la peroxydase et la catalase ont été mesurés à l'aide de méthodes standard. Des exsudats racinaires ont été collectés pour l'analyse des acides organiques, c.-à-d. l'acide citrique et l'acide tartrique. L'alcalinité de la cendre de racine a aussi été mesurée. Les résultats indiquent que la salinité cause une augmentation significative de l'activité des enzymes antioxydantes et la libération d'acides organiques chez les deux espèces. Avec l'augmentation de l'activité des enzymes antioxydantes il y avait moins de dommages aux membranes et une plus grande production de biomasse racinaire et caulinaire chez *A. ampliceps* que chez *A. nilotica*. De même, l'acidification plus prononcée de la rhizosphère a permis à *A. ampliceps* de mieux réagir à la salinité que sa contrepartie. [Traduit par la Rédaction]

Mots-clés : salinité, alcalinité des cendres, acide citrique, catalase, peroxydase.

Introduction

Salinity is one of the major abiotic stresses and has a large impact on plant growth and productivity. It is estimated that 6% of the world's land and 30% of the world's irrigated areas are suffering from salinity problems (Munns and Tester 2008). Salinity stress causes many morphological, physiological, and biochemical disorders in plants, including reductions in shoot and root growth (Zhang et al. 2013), photosynthesis, respiration (Marschner 1995), and in the activities of various enzymes (Sairam and Tyagi 2004). The extent of damage depends on the duration of stress, plant species (Nawaz et al. 2010), type of salts, and the plant's growth stage (Lauchli and Grattan 2007).

Due to osmotic stress caused by salinity, stomata are closed, leading to a reduced supply of CO_2 for photosynthesis and an overproduction of various reactive oxygen species (ROS) (Ashraf 2009). Among these ROS, hydrogen peroxide, superoxides, and hydroxyl radicals are mainly produced in the cytosol, chloroplasts, mitochondria, and apoplastic spaces (Shahid et al. 2014). These ROS cause very harmful effects in the cell such as lipid peroxidation, protein damage, and changes in the DNA molecule (Shahid et al. 2012). Membrane injury due to salinity is also related to the overproduction of these ROS (Shalata et al. 2001).

These ROS are detoxified with the help of various enzymes and nonenzymes. The enzymes include superoxide dismutase (SOD), peroxidise (POD), catalase (CAT), and those of the ascorbate-glutathione cycle (Pourrut et al. 2011). The nonenzyme antioxidants include tocopherols, flavones, ascorbic acid, and carotenoids (Johnson et al. 2003). Superoxides are converted to hydrogen peroxide and molecular oxygen with the help of SOD (Giannopolitis and Ries 1977). SOD is an important enzyme for mitigating oxidative stress in plants. Hydrogen peroxide and some organic hydroperoxides are converted to water and oxygen with the help of CAT and POD (Ali and Alqurainy 2006). The activities of enzymes and nonenzymes of different plant species under salinity stress vary greatly (Nayyar and Gupta 2006). Plants with a higher antioxidant production capacity are thought to be more salt tolerant (Mittal et al. 2012).

The release of hydrogen ions (H^+) from plant roots is thought to lower the pH of the rhizosphere (Shahid et al. 2012). Leguminous

Received 7 August 2014. Accepted 3 October 2014.

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plants have been shown to acidify their rhizospheres (Schubert et al. 1990*a*) by releasing a sufficient amount of H^+ from their roots (Schubert et al. 1990*b*). This H^+ extrusion is responsible for the increase in pH of the cytosol, which in turn hastens the formation of organic anions. These organic anions are a measure of how many hydrogen ions are released in the rhizosphere, and an estimate of these organic anions is called the ash alkalinity. This parameter has been characteristically determined in many plants to evaluate their acidification capacity (Moody and Aitken 1997). It is hypothesized that oxidative-stress tolerance and rhizosphere acidification will positively correlate with salinity tolerance of different acacia species. The objective of this study was to investigate the effect of salt on the activities of the antioxidant enzymes and the release of various organic anions in two acacia species, i.e., *Acacia nilotica* L. and *Acacia ampliceps* Maslin. The former species was chosen because it is local, well acclimatized to the arid climatic conditions of Pakistan, and moderately tolerant to salinity stress (Abbas et al. 2013). The latter species belongs to Australia (exotic to Pakistan) and is not well acclimatized to the arid climatic conditions in Pakistan; however, it is highly tolerant to salinity (Abbas et al. 2013) and has the potential of growth in Pakistani conditions. The effect of salinity on antioxidant enzymes has not been determined in these species; moreover, the determination of rhizosphere acidification potential in local and exotic acacia species adapted to saline sodic conditions might be helpful in identifying the plants with a greater capacity for dissolution of calcite by releasing more H^+ in their rhizospheres, thus improving the quality of the salt-affected soils.

Materials and methods

Growth conditions and experimental techniques

This experiment was conducted in a greenhouse at the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan. The mean monthly data for weather conditions were as follows: minimum temperature, 15 °C; maximum temperature, 36 °C; minimum relative humidity, 39%; maximum relative humidity, 65%; and daily duration of sunshine, 8 h and 40 min. The healthy nursery of three-week-old *A. ampliceps* and *A. nilotica* plants was transferred to Hoagland's nutrient solution. There were four replications of each treatment, with one plant in each replication. One week after transplantation, the plants were exposed to NaCl concentrations of 100 and 200 mmol·L^{-1}. The pH of the solution was monitored daily and maintained at 6.0 ± 0.5 throughout the experiment by the addition of NaOH or HCl. After four weeks of salinity stress, plants were harvested, and shoot and root fresh masses and their respective lengths were noted. The shoots and roots were oven-dried separately at 65 °C for 48 h, and their dry masses were recorded.

Determination of the membrane stability index (MSI) and ash alkalinity

Under salt-stress conditions, the permeability of plant membranes is increased, which causes the membrane integrity to be lost. The degree of stress-induced membrane injury may be easily estimated by measuring the electrolyte leakage from the cells. A higher amount of electrolyte leakage indicates a lower plant MSI. The MSI was determined by estimating how many ions leached from leaf tissues into distilled water, following the method of Sairam et al. (2002).

When plants uptake cations, the extrusion of H^+ is enhanced due to fractional depolarization of the membrane potential, which further helps in active H^+ pumping. This H^+ extrusion increases the pH of the cytosol, which in turn increases ash akalinity. This parameter has been characteristically determined in many crops and trees to evaluate their acidification capacity. In this study, ash alkalinity was measured following the method of Jungk (1968).

Table 1. Effect of salinity stress on growth parameters of acacia species.

	Control	100 mmol·L^{-1} NaCl	200 mmol·L^{-1} NaCl
Shoot fresh mass (g·plant^{-1})			
A. ampliceps	2.40±0.08a	2.04±0.03b	1.55±0.05d
A. nilotica	2.31±0.05a	1.76±0.07c	1.05±0.04e
Shoot dry mass (g·plant^{-1})			
A. ampliceps	0.42±0.01a	0.36±0.01b	0.28±0.02c
A. nilotica	0.41±0.01a	0.30±0.02c	0.20±0.01d
Root fresh mass (g·plant^{-1})			
A. ampliceps	1.11±0.03a	1.00±0.04a	0.72±0.02c
A. nilotica	1.03±0.04a	0.82±0.03b	0.51±0.04d
Root dry mass (g·plant^{-1})			
A. ampliceps	0.18±0.004a	0.16±0.005b	0.12±0.004c
A. nilotica	0.18±0.003a	0.14±0.04c	0.09±0.002d
Shoot length (cm)			
A. ampliceps	18.5±0.46b	16.4±0.41c	11.4±0.61d
A. nilotica	20.5±0.29a	17.6±0.41bc	11.5±0.41d
Root length (cm)			
A. ampliceps	13.9±0.40b	12.4±0.24c	8.93±0.39d
A. nilotica	15.7±0.47a	13.3±0.46bc	9.22±0.71d

Note: Values are mean ± SE of four replicates. Means that share the same lowercase letter are statistically nonsignificant at $p \leq 0.05$.

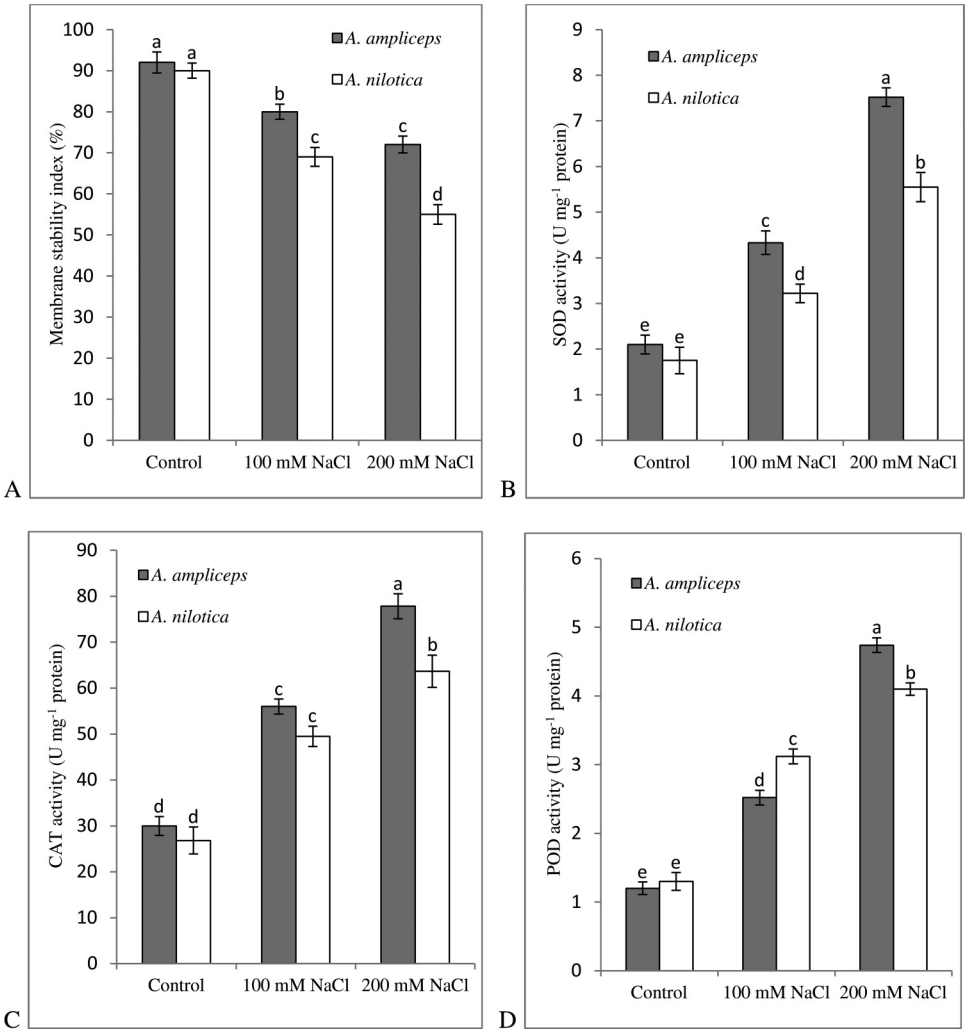
Determination of activities of antioxidant enzymes

For extracting antioxidant enzymes, 0.5 g fresh leaf samples were ground using a tissue grinder in 5 mL of 50 mmol·L^{-1} cold phosphate buffer (pH 7.8) and placed in an ice bath. The homogenate was centrifuged at 15 000 g for 20 min at 4 °C, and the supernatant was used for determination of antioxidant enzymes. SOD activity was determined by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT), using the method as described by Giannopolitis and Ries (1977). The reaction mixture (3 mL) contained 50 μmol·L^{-1} NBT, 13 mmol·L^{-1} methionine, 1.3 μmol·L^{-1} riboflavin, 50 mmol·L^{-1} phosphate buffer (pH 7.8), 75 nmol·L^{-1} EDTA, and 20–50 μL of enzyme extract. The test tubes containing the reaction solution were irradiated under light (15 fluorescent lamps) at 78 μmol·m^{-2}·s^{-1} for 15 min. The absorbance of the irradiated solution was recorded on a UV–Visible spectrophotometer at 560 nm. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction. The method of Chance and Maehly (1955) was adopted to determine the activities of CAT and POD. The CAT reaction mixture (3 mL) contained 5.9 mmol·L^{-1} H_2O_2, 50 mmol·L^{-1} phosphate buffer (pH 7.0), and 0.1 mL of enzyme extract. The reaction was initiated by the addition of the enzyme extract. The changes in absorbance of the reaction mixture at 240 nm were recorded every 20 s. The POD reaction mixture (3 mL) contained 20 mmol·L^{-1} guaiacol, 50 mmol·L^{-1} phosphate buffer (pH 5.0), 40 mmol·L^{-1} H_2O_2, and 0.1 mL of enzyme extract. The changes in absorbance of the reaction mixture at 470 nm were recorded after every 20 s. One unit of enzyme activity was defined as the change in absorbance of 0.01 units·min^{-1}.

Root-exudates collection and analysis

The collection of root exudates and their analysis for organic acids was done following the method of Fang et al. (2008), with some changes in the chromatographic conditions. The root exudates were collected after two weeks of the salt treatment. After 2 h of exposure to light in the morning, the plants were taken from the solution and washed three times with distilled water. The plant roots were placed in a conical flask containing 100 mL of deionized water. After 2 h, the plants were returned to the nutrient solution. The exudates solution was then filtered and concentrated with the help of a rotary evaporator. The root exudates were redissolved in 10 mL of deionized water and transferred to test tubes. Prior to the sample measurement, the root-exudates

Fig. 1. Effect of salinity stress on (A) membrane stability index (MSI), (B) superoxide dismutase (SOD) activity, (C) catalase (CAT) activity, and (D) peroxidase (POD) activity of *A. nilotica* and *A. ampliceps*. Vertical bars indicate mean ± SE of four replicates. For both species, values sharing a common lowercase letter are nonsignificant at $p \leq 0.05$. mM, mmol·L^{-1}.



solution was filtered with a 0.45 mm filter film. High-pressure liquid chromatography (HPLC-10A, Shimadzu Scientific Instruments) was used to isolate the organic acids in the root exudates and determine their concentrations. The chromatographic conditions were as follows: column, Shim-Pack CLC-ODS (C-18) (25 cm × 4.6 mm, 5 μm); mobile phase, isocratic H_3PO_4 (6×10^{-3} mol·L^{-1}, pH 2.1); injection volume, 20 mL; flow rate, 0.7 mL·min^{-1}; column temperature, room temperature; detectors, SPD 10-A and UV–Visible spectrophotometer at 210 nm. Determination of organic acids was done through the standard reagent method, and the concentrations of these organic acids were determined by the peak-area method.

Statistical analysis

The experimental data were subjected to statistical analysis in a completely randomized design with factorial arrangements (Steel et al. 1997). The significance of differences among treatments and genotypes were determined using a least significant difference (LSD) test.

Results

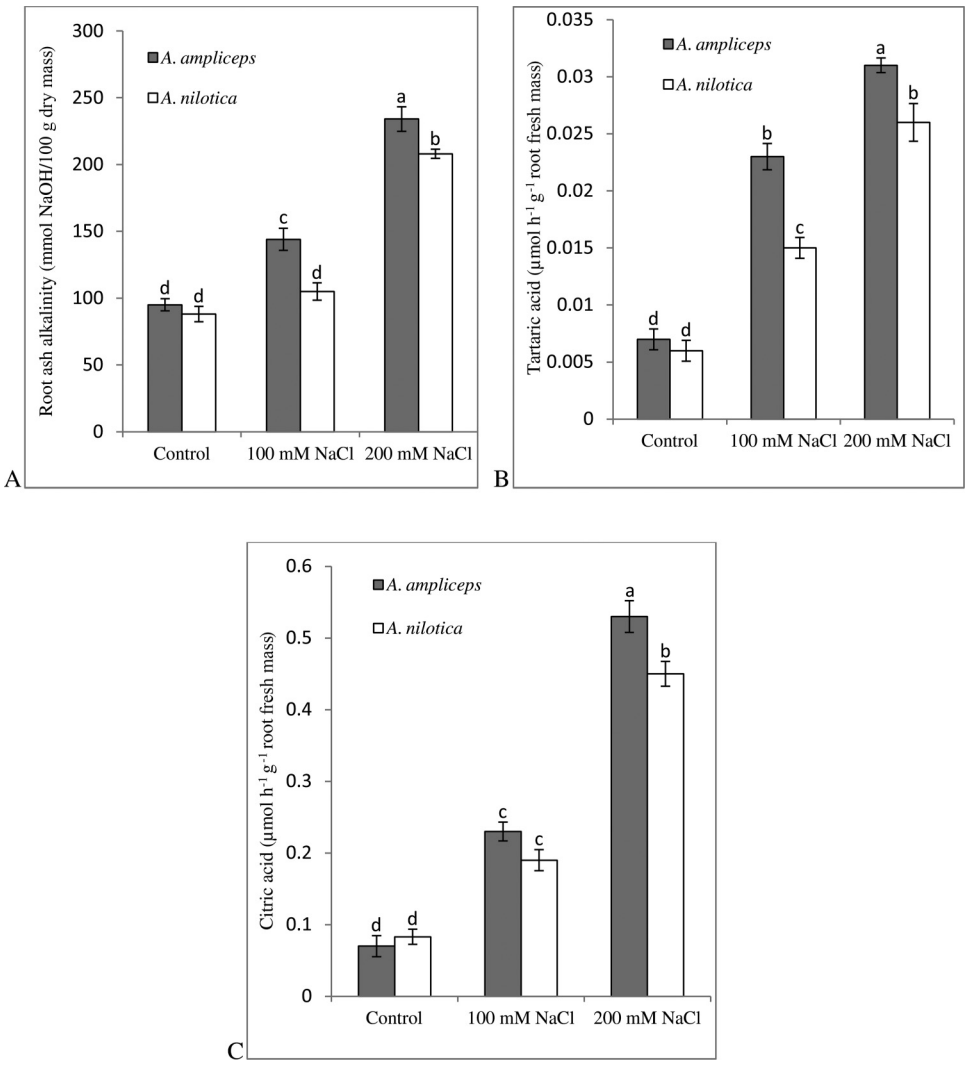
Shoot and root growth

Shoot fresh and dry masses were reduced in response to salinity in both acacia species (Table 1). The differences among species and treatments and their interaction were significant ($p \leq 0.05$) for both these parameters. The reduction in shoot fresh and dry mass was greater for *A. nilotica* than for *A. ampliceps* under both salinity levels. There was a greater difference between species at the NaCl concentration of 200 mmol·L^{-1} than that of 100 mmol·L^{-1}. Both root fresh and dry masses decreased with increasing NaCl concentration in the growing medium (Table 1). The main effects, as well as their interaction, were significant ($p \leq 0.05$) for both root fresh and dry masses. There was a greater decrease in root fresh and dry masses for *A. nilotica* than for *A. ampliceps* under both salt treatments. The shoot and root lengths (cm) also decreased due to salt stress (Table 1). Although both treatments and species had significant effects, the species–treatment interaction was found to be nonsignificant ($p \leq 0.05$) for both shoot and root lengths. *Acacia nilotica* produced more shoot and root length than *A. ampliceps* under nonsaline conditions. However, under saline conditions, the species did not differ significantly from each other.

MSI and the activities of antioxidants

The increasing salt concentrations in the growing medium caused oxidative damage to the cell membranes, indicated by the decrease in the MSI value (Fig. 1A). The effects of treatment and species and their interaction were significant ($p \leq 0.05$). *Acacia ampliceps* showed a significantly higher MSI value than *A. nilotica*

Fig. 2. Effect of salinity stress on (A) root ash alkalinity, (B) tartaric acid, and (C) citric acid of *A. nilotica* and *A. ampliceps*. Vertical bars indicate mean ± SE of four replicates. For both species, values sharing a common lowercase letter are nonsignificant at $p \leq 0.05$. mM, mmol·L^{-1}.



under both stress treatments. To mitigate this oxidative damage, the activities of antioxidant enzymes were increased as the salt levels increased in the growing medium. The activity of SOD increased in both species in response to the increase in salinity, and this increase was higher in *A. ampliceps* than in *A. nilotica* (Fig. 1B). Regarding SOD, the individual and the species–treatment interaction effects were found to be significant ($p \leq 0.05$). The activity of CAT also increased with the increase in salinity for both species; however, the species–treatment interaction was found to be nonsignificant ($p \leq 0.05$) (Fig. 1C). Both species only differed at the NaCl concentration of 200 mmol·L^{-1}, with *A. ampliceps* showing more CAT activity than *A. nilotica*. Similar to the other enzymes, the activity of POD also increased in response to the increase in salinity for both species (Fig. 1D). In this case, the species effect was nonsignificant ($p \leq 0.05$). At the lower salinity level (100 mmol·L^{-1} NaCl), POD activity levels were significantly higher for *A. nilotica*. On the other hand, at the higher salinity level (200 mmol·L^{-1} NaCl), *A. ampliceps* had more POD activity than *A. nilotica*.

Ash alkalinity and organic acids

For both species, a significant increase was observed in root ash alkalinity in response to salinity stress (Fig. 2A). The species–treatment interaction was found to be nonsignificant ($p \leq 0.05$). The ash alkalinity was significantly higher in *A. ampliceps* than in *A. nilotica* under both salinity levels. Regarding organic acids, it was observed that with an increase in salinity levels, the release of these organic acids was also increased. For tartaric acid, the individual and species–treatment interaction effects were significant ($p \leq 0.05$). *Acacia ampliceps* released significantly more tartaric acid than *A. nilotica* under both salinity levels (Fig. 2B). It was noticed that the release of citric acid was higher for *A. ampliceps* than for *A. nilotica* (Fig. 2C). The individual and species–treatment effects were found to be significant ($p \leq 0.05$). The difference between both species was nonsignificant at the NaCl concentration of 100 mmol·L^{-1}; however, their difference was significant at the NaCl oncentration of 200 mmol·L^{-1}.

Discussion

The results of all of the studied parameters indicated that there was a distinct difference between both species regarding their response to salinity stress. Fresh and dry masses of both shoot and root decreased in both species; however, *A. nilotica* showed more reduction in these parameters than *A. ampliceps*, which matches the findings of Abbas et al. (2013). Moreover, our results are in accordance with the findings of Hardikar and Pandey (2008) for *Acacia senegal* (L.) Willd. and Marcar and Crawford (2011) for *A. ampliceps*. The shoot and root lengths also decreased due to

salinity stress. *Acacia nilotica* produced more shoot and root length than *A. ampliceps* under nonsaline conditions; however, under saline conditions, there was no significant different between the species. This shows that the relative decrease in both shoot and root lengths was greater for *A. nilotica* than for *A. ampliceps*. The reduction in acacia shoot and root lengths due to salinity stress was previously observed by Abbas et al. (2013) and Al-Shaharani and Shetta (2011). Such reductions in plant growth parameters under saline conditions may be attributed to the osmotic and ionic effects of salts (Nawaz et al. 2010). These effects were less harmful for *A. ampliceps* due to its higher tolerance of and better adaptation to saline conditions. Although it is an exotic species, it would be a more promising option than *A. nilotica* for the rehabilitation of salt-affected barren lands due to its better growth and biomass production.

Membrane injury due to salinity stress is related to the overproduction of ROS (Shalata et al. 2001). The MSI decreased in response to salinity stress, indicating the oxidative damage and toxic effects of salt on the structure of the membranes. *Acacia ampliceps* had a significantly greater MSI value than *A. nilotica* under both stress treatments, indicating higher salinity tolerance and less membrane damage than *A. ampliceps*. To mitigate this oxidative damage, the activities of antioxidant enzymes increased with increasing salt stress. In the present study, SOD activity increased under salt stress. This enzyme dismutates superoxide radicals to H_2O_2 and O_2 in the cytosol, mitochondria, and chloroplast (Giannopolitis and Ries 1977; Shahid et al. 2014). Increased activities of SOD under salt stress conditions were also observed by Mittal et al. (2012), indicating its role in salinity tolerance. Chen et al. (2011) observed that a salt-tolerant wheat cultivar exhibited higher SOD activity compared with a sensitive cultivar. These results match our findings, as we also found higher SOD activity in *A. ampliceps*, which is more salt tolerant than *A. nilotica*, especially at a NaCl concentration of 200 mmol·L^{-1}. It can be concluded that SOD may have a crucial role in modulating the salinity tolerance of the two acacia species. CAT and POD are involved in the detoxification of H_2O_2 (Pourrut et al. 2011), and the activities of both these enzymes increased in both species, with greater increase in *A. ampliceps* at a NaCl concentration of 200 mmol·L^{-1}. The higher activity of CAT and POD seems to be responsible for the detoxification of H_2O_2, more so for *A. ampliceps* than for *A. nilotica*. Morais et al. (2012) conducted a three-month experiment under controlled conditions with four concentrations of NaCl (0, 50, 100, and 200 mmol·L^{-1}) and found that *Acacia longifolia* (Andr.) Willd. was better adapted to saline conditions than *Ulex europaeus* L., owing to its better antioxidant activities particularly of CAT. Some other researchers also found increased activities of these enzymes in response to salinity stress such as Patel and Saraf (2013) and Zhang et al. (2013). The better growth of *A. ampliceps* might be due to its better detoxification capacity against ROS, which enabled it to withstand the saline environment better than *A. nilotica*. Both these species have the capacity to grow on salt-affected soils; however, the higher antioxidant activity of *A. ampliceps* makes it more suitable for growing on such soil conditions. Exploring this salinity tolerance mechanism might be helpful for the identification of other species for their suitability for growing on salt-affected lands.

The root ash alkalinity increased in both species in response to salinity stress and was more important for *A. ampliceps* than for *A. nilotica*. Ash alkalinity is a measure of how many hydrogen ions are released from the plants (Jungk 1968). In case of *A. ampliceps*, more ash alkalinity indicated the higher acidification potential of this species compared with *A. nilotica*. Legume plants, dependent on nitrogen fixation, uptake more cations than anions and, therefore, release more hydrogen ions in their rhizospheres (Tang et al. 2001). However, there is a large difference within the species regarding proton release. With an increase in salinity, the release of organic anions, i.e., citric acid and tartaric acid, also increased. The findings of Chen and Lin (2010) are in accordance with our results. They found increased concentrations of various organic acids in tomato roots in response to NaCl stress and relate those concentrations with the salinity tolerance of tomatoes. The release of organic acids is considered to be one of the crop's stress-tolerance mechanisms (Larsen et al. 1998). Some special types of proteins are involved in organic-acid release under stress conditions (Ryan et al. 1995). According to Qadir et al. (2005), rhizosphere acidification may be helpful in reducing soil salinity and sodicity by dissolving native lime and hence positively correlate with the salinity tolerance of different plant species. We also observed that *A. ampliceps* released more organic acids and had a higher ash alkalinity than *A. nilotica*; therefore, *A. ampliceps* can grow better under salt-affected conditions. Based on this study, it can be concluded that the increased release of antioxidants and organic acids has a crucial role in the growth and survival of plants under saline conditions. Moreover, the reclamation of salt-affected lands by growing trees, particularly trees such as *A. ampliceps*, would be a long-term, sustainable approach.

Acknowledgement

The financial support for the chemicals and instrumentation was provided by the Endowment Fund Secretariat, University of Agriculture, Faisalabad, Pakistan.

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