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Applications of ascorbic acid or proline increase resistance to salt stress in barley seedlings

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Abstract

The present study was carried out to examine the effects of seed soaking in 1 mM ascorbic acid (AA) or 1 mM proline on the growth, content of photosynthetic pigments and proline, relative water content, electrolyte leakage, antioxidant enzymes and leaf anatomy of *Hordeum vulgare* L. Giza 124 seedlings grown in greenhouse under 100 or 200 mM NaCl. The plants exposed to the NaCl stress exhibited a significant reduction in growth, relative water content, leaf photosynthetic pigments, soluble sugars, as well as alterations in leaf anatomy. However, the treatment with AA or proline ameliorated the stress generated by NaCl and improved the above mentioned parameters. NaCl increased electrolyte leakage, proline content, and activities of antioxidant enzymes (SOD, CAT, and POX). The antioxidant enzymes and leaf anatomy exhibited considerable changes in response to AA or proline application in the absence or presence of NaCl.

Additional key words: carotenoids, catalase, chlorophyll, electrolyte leakege, *Hordeum vulgare*, leaf anatomy, NaCl, peroxidase, relative water content, superoxide dismutase.

Introduction

High salinity in soils inhibiting crop growth and yield is a frequent constraint to agriculture in arid and semi-arid regions. Increasing Na^+ and Cl^- content in plant tissue can increase oxidative stress, which causes deterioration in chloroplast ultrastructure and even loss in chlorophyll (Khosravinejad and Farboondia 2008). Cucumber seedlings exposed to 75 mM NaCl for 5 d show reduced root and leaf dry masses, leaf area, and net CO_2 assimilation rate (Zhen *et al*. 2012). High salinity results in hyperosmolarity, ion disequilibrium, nutrient imbalance, and production of reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide and hydoxyl radical (Nawaz *et al*. 2010), which are responsible for the damage to membranes, proteins, DNA, and lipids. Various changes in activities of various antioxidant enzymes under salinity stress have been reported (Dolatabadian and Saleh 2009). For example, a salt stress increases a catalase (CAT) activity, whereas a superoxide dismutase (SOD) activity is not changed in cashew leaves (Ferreira-Silva *et al*. 2012). The induction of salt tolerance in plants is crucial to maintain their economic yield. This can be achieved either through genetic modifications or by chemical treatments (Hamdia and Shaddad 2010). The strategies of plant breeding and genetic engineering are long-term and complex endeavours to develop salt tolerance that still has limited success (Ashraf *et al.* 2008). Alternatively, the application of non-enzymatic antioxidants, such as ascorbic acid, is an efficient technique to cope with the deleterious effects of salinity on plants (Khan *et al.* 2006). During stress, the endogenous content of growth regulators is often lowered which can be overcome by their application. Also, the application of fertilizers and plant growth regulators has been successfully used to minimize the adverse effects of salinity on plant growth and yield (Tuna *et al.* 2008, Ashraf *et al.* 2010, Kaya *et al.* 2010).

Ascorbic acid (AA) is regarded as one of the most effective compounds able to ameliorate abiotic stresses (Conklin 2001). AA not only acts as an antioxidant but it activates a complex of defence mechanisms (Conklin and Barth 2004). It has been used to counteract the adverse effects of salt stress in many crop plants (Bassuony *et al.* 2008, Beltagi 2008, Khan *et al.* 2010).

One of the most common stress responses in plants is overproduction of different types of compatible organic solutes (Serraj and Sinclair 2002) which can contribute to cellular osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of

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Abbreviations: AA - ascorbic acid; Car - carotenoids; CAT - catalase; Chl - chlorophyll; EL - electrolyte leakage; POD - peroxidase; RWC - relative water content; SOD - superoxide dismutase.
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enzymes/proteins (Yancey *et al.* 1982, Bohnert and Jensen 1996). These solutes include proline, sucrose, trehalose, polyols, and many others (Rhodes and Hanson 1993). Proline is one of the well-known osmoprotectants and its accumulation is widely observed in various organisms under salt stress. Proline content is dependent on its synthesis, catabolism, and transport from other tissues. Further, foliar spray of proline or glycinebetaine counteracted the growth inhibition induced by NaCl in rape seeds (Makela *et al.* 1999), rice (Rahman *et al.* 2002, Roy *et al*., 1993), wheat (Raza *et al.* 2006), and maize (Ali *et al.* 2007). The proline application effectively regulates osmotic potential and plays a vital role in sustaining plant growth under an osmotic stress (Serraj and Sinclair 2002, Ali *et al.* 2007, Ashraf and Foolad 2007, Hoque *et al.* 2007).

Barley growth and development is greatly affected by salt stress (Hussain *et al.* 1997, Mer *et al.* 2000). The present study was designed to evaluate whether the adverse effects of a NaCl stress on barley seedling growth, leaf anatomy, some metabolites, and antioxidant system could be mitigated by pretreatments with ascorbic acid and proline.

Materials and methods

Two pot experiments were conducted at the Faculty of Agriculture, Southeast Fayoum, Egypt, during two successive seasons. Seeds of barley (*Hordeum vulgare* L. Giza 124) were obtained from the Agricultural Research Center, Egypt. Seeds of a uniform size were washed with distilled water after surface sterilizing with a 10 % sodium hypochlorite solution. Then seeds were imbibed in water, 1 mM ascorbic acid (AA), or 1.0 mM proline for 12 h. The selections of the concentrations and the duration of soaking were based on a preliminary experiment (data not shown). On the 15th November 2011 and 15th November 2012 the seeds were sown in plastic pots (20 cm in diameter) equally filled with sand moistened with distilled water and grown in a greenhouse under day/night temperatures of 20 ± 5/10 ± 2 °C, relative humidities of 82/63 %, and natural irradiance with a photoperiod of 10 to 11 h. At the third leaf stage, the seedlings were supplemented with NaCl (0.0, 100, or 200 mM) along with a half-strength Hoagland's nutrient solution. The seedlings were irrigated with the Hoagland's solution every 3 d throughout the experiment. Samples of leaves were collected at 49 d after sowing to assess content of chlorophylls, carotenoids, proline, and soluble sugars, electrolyte leakage, relative water content, and activities of antioxidant enzymes. At the end of the experiment (56 d after sowing), the plants were removed from the pots, washed in water, and the lengths of shoots and roots were measured. A number of leaves was counted and a leaf area was recorded by a *LI-3000* portable area meter (*LI-COR*, Lincoln, NE, USA). The plants were weighed to record a plant fresh mass and then placed in an oven run at 80 °C for 24 h. The dried plants were weighed to record a plant dry mass.

Leaf chlorophylls and carotenoids were determined in acetone extracts according to Arnon (1949) using a spectrophotometer (Schimadzu, Kyoto, Japan). Leaf soluble sugars were assessed by the method recommended by the Association of Official Agricultural Chemists (1990) using phenol sulphuric acid reagent method. A proline content was measured by the rapid colorimetric method as suggested by Bates *et al*. (1973). The total inorganic ions leaked out from the leaf tissues were determined by a digital conductivity meter (*Inolab*, Weilheim, Germany) following Dionisio-Sese and Tobita (1998). For determination of electrolyte leakage (EL), 20 leaf discs were put in a tube containing 10 cm^3 of deionized water and electrical conductivity was measured (EC0) after 2 h. The tube was heated to 50 - 60 °C in a water bath and after 25 min EC was measured (EC1). Then, the content was boiled at 100 °C for 10 min and the EC was again recorded (EC_2). EL was calculated using the formula: $EL\ [\%] = (EC_1 - EC_0) / (EC_2 - EC_0) \times 100$.

A relative water content (RWC) was estimated as described by Yamasaki and Dillenburg (1999). The fourth leaves from an apex (to minimize the age effect on the variability of results) were cut, weighed (FM), and floated in distilled water in closed Petri plates and weighed periodically till their full saturation with water (WSM). At the end, they were placed in an oven at 80 °C for 48 h to obtain dry mass (DM), and RWC was calculated as: $RWC\ [\%] = [(FM - DM) / (WSM - DM)] \times 100$.

For antioxidant enzyme activity determinations, 1.0 g of fresh leaves was ground in an ice-cold mortar with a pestle with 10 cm^3 of a 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 20 000 *g* and 4 °C for 30 min. The supernatant filtered through two layers of cheese-cloth was used for the assays of enzymatic activities. The SOD (EC: 1.15.1.1) activity was determined according to the method of Fridovich (1975). One unit of the SOD activity was defined as the amount of the enzyme required to cause 50 % inhibition in the rate of nitroblue tetrazolium (NBT) photooxidation measured spectrophotometrically at 560 nm. The CAT (EC: 1.11.1.6) activity was determined by employing the method suggested by Luck (1975) estimating the residual H_2O_2 by oxidation with $KMnO_4$ titrimetrically. The POD (EC: 1.11.1.7) activity was estimated using the method of Thomas *et al*. (1981) using guaiacol as the substrate. The absorbance was measured at 436 nm. One unit of POD activity was defined as the amount of enzyme which caused a change of 0.01 in absorbance per min.

For observation of leaf anatomy, samples were taken from the apex of the fourth leaf and fixed in FAA solution (containing 50 cm^3 of 95 % (v/v) ethanol + 10 cm^3 of formaldehyde + 5 cm^3 of glacial acetic acid + 35 cm^3 of distilled water) for 48 h. Thereafter, the

samples were washed in 50 % ethanol, dehydrated and cleared in tertiary butanol series, and embedded in paraffin wax. Cross sections, 25 μm thick, were cut by a rotary microtome (*Leitz*, Wetzlar, Germany), adhered by a Haupt's adhesive, stained with a crystal violet-erythrosin combination (Sass 1961), cleared in carbol xylene, and mounted in Canada balsam. The sections were observed and documented using an upright light microscope (*AxioPlan*, Zeiss, Jena, Germany). Measurements were done using a micrometer eyepiece and average of five readings was calculated.

All the pots (261) were arranged in a complete randomized design with six pots per replicate and four replicates per treatment. Analysis of variance was performed using the *SPSS* software package to determine the least significant difference (LSD) among treatments at $P \leq 0.05$, and the Duncan's multiple range tests were applied for comparing the means.

Results

An increase in NaCl concentrations to 100 or 200 mM significantly reduced the length of shoot, number of leaves, leaf area per plant, and plant fresh and dry masses (Table 1). Compared to the control, the above mentioned parameters were decreased under 200 mM NaCl by 44.6, 28.5, 56.9, 90.1, and 83.5 %, respectively. After the treatment of the barley seeds with AA or proline and in absence of the NaCl stress, the growth was generally higher than that of the control ($P \leq 0.05$). AA or proline also improved the growth of the plants grown under the NaCl stress and the values in most cases were significantly higher than those of the plants grown under the stress alone. AA was more efficient than proline in this concern.

The NaCl stress significantly decreased the content of chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car) (Table 2). The 200 mM NaCl was more toxic compared to the 100 mM one and the decrease compared to the control reached 48.6, 50.0, and 17.2 % for Chl *a*, Chl *b*, and Car, respectively. However, soaking the barley seeds in AA or proline increased the content of Chl *a*, Chl *b*, and Car especially in the absence of the NaCl stress. The AA treatments were more effective than the proline ones.

An increase in salinity significantly reduced the total soluble sugar content of the barley leaves (Table 2). At 100 and 200 mM NaCl, the decrease was 38.6 and 47.1 %, respectively, in comparison with the control. Soaking the seeds in AA or proline increased the content of total soluble sugars in the presence or absence of the NaCl stress. The amount of proline in leaves significantly increased in response to the NaCl stress and the maximum was found in the plants subjected to 200 mM NaCl. Soaking the seeds in AA did not bring about a significant change in the proline content of the unstressed

Table 1. Effects of 1 mM ascorbic acid (AA) or 1 mM proline (Pro) applications on the shoot length [cm], number of leaves [plant^{-1}], leaf area [cm^2 plant^{-1}], fresh mass [g plant^{-1}], and dry mass [g plant^{-1}] of barley plants grown under the 100 or 200 mM NaCl stress. Means ± SD, $n = 5$. Mean values in each column followed by different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range test.

Parameters	0 (control)	100	200	0 + AA	100 + AA	200 + AA	0 + Pro	100 + Pro	200 + Pro
Shoot length	47.0±1.0b	32.7±0.3e	26.0±1.0f	50.0±1.5a	42.8±0.6c	36.3±0.6d	49.6±1.5a	45.7±0.4b	36.6±1.1d
Leaf number	5.6±0.6a	4.3±0.6bc	4.0±0.0c	6.0±0.0a	5.3±0.6ab	5.0±1.0abc	5.6±0.6a	5.3±0.6ab	5.0±0.0abc
Leaf area	20.9±0.4bc	11.9±0.2ef	9.0±0.3f	26.0±1.0a	17.1±0.5cd	14.8±0.7de	24.3±0.8ab	17.7±1.0cd	14.2±0.4de
Plant FM	8.5±0.3b	1.4±0.0e	0.8±0.0f	10.6±0.4a	5.2±0.1c	4.7±0.0c	10.4±1.0a	4.0±0.0d	3.7±0.1d
Plant DM	1.7±0.1c	0.5±0.0f	0.3±0.0g	2.1±0.0a	1.2±0.0d	1.1±0.0e	1.9±0.1b	1.1±0.0e	1.1±0.0e

Table 2. Effects of 1 mM ascorbic acid (AA) or 1 mM proline (Pro) appplications on the content of Chl *a*, Chl *b*, Car, total soluble sugars, and endogenous proline [mg g^{-1}(FM)] in leaves of barley plants grown under the 100 or 200 mM NaCl stress. Means ± SD, $n = 5$. Mean values in each column followed by different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range test.

Parameters	0 (control)	100	200	0 + AA	100 + AA	200 + AA	0 + Pro	100 + Pro	200 + Pro
Chl *a*	1.09±0.09b	0.86±0.26d	0.56±0.01e	1.65±0.06a	0.97±0.01c	0.81±0.01d	1.15±0.02b	1.11±0.05b	1.08±0.04b
Chl *b*	0.30±0.00b	0.25±0.00d	0.15±0.00f	0.34±0.00a	0.27±0.00c	0.23±0.00e	0.27±0.00c	0.33±0.00a	0.29±0.00b
Car	0.29±0.00e	0.24±0.00f	0.24±0.01f	0.38±0.00a	0.32±0.00d	0.25±0.00f	0.36±0.00b	0.34±0.01c	0.37±0.00ab
Sugars	38.60±0.23b	23.70±0.37f	20.40±0.33g	44.70±0.31a	30.10±0.13c	28.40±0.45e	38.70±0.23b	30.10±0.16c	28.90±0.51d
Proline	0.13±0.00g	2.40±0.26d	4.00±0.07a	0.12±0.01g	1.80±0.00f	2.10±0.01e	0.32±0.00g	2.80±0.14c	3.70±0.13b

plants, but as was expected, soaking the seed in proline increased the leaf proline content. However, in association with NaCl, AA or proline significantly reduced the proline content in leaves compared to plants subjected to 200 mM NaCl alone.

Increasing NaCl concentration led to a significant decrease in RWC (Table 3). Soaking the seeds in AA or proline improved the RWC in presence of NaCl. The NaCl stress increased EL as compared to the control and maximum EL was recorded in the plants exposed to 200 mM NaCl. Under unstressed conditions AA or proline only slightly affected EL but decreased it under the stress.

The antioxidative enzyme activities increased in response to the NaCl stress (Table 3). The AA or proline pretreatments caused a significant increase in the activities of SOD and CAT but the POD activity was only slightly increased as compared to the control. Thus, maximum activities of these enzymes were recorded in the plants pre-treated with AA or proline and exposed to 200 mM NaCl.

As concerns the leaf anatomical structure, the thickness of midvein, blade, mesophyll, adaxial and abaxial epidermes, and average diameter of vessels were decreased in response to 200 mM NaCl by 29.8, 41.9, 45.8, 25.0, 33.3, and 31.8 %, respectively, in comparison to the control. However, soaking the seeds in AA or proline caused positive changes in the above mentioned characteristics in the absence or presence of the NaCl stress. For example, the maximum midvein thickness was observed in the AA pretreated plants in absence of the NaCl stress as compared to other treatments.

Table 3. Effects of 1 mM ascorbic acid (AA) or 1 mM proline (Pro) applications on RWC [%], EL [%], and the activities of SOD [U g^{-1}(FM)], POD [U g^{-1}(FM)], and CAT [mmol(H_2O_2) g^{-1}(FM) min^{-1}] in the leaves of barley plants grown under the 100 or 200 mM NaCl stress. Means ± SD, n = 5. Mean values in each column followed by different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range test.

Parameters	0 (control)	100	200	0 + AA	100 + AA	200 + AA	0 + Pro	100 + Pro	200 + Pro
RWC	76.40±0.26c	52.20±0.17h	50.20±0.11i	88.60±0.15b	67.70±0.47e	64.20±0.20g	89.70±0.32a	69.10±0.96d	65.70±0.20f
EL	10.10±0.11f	11.80±0.05b	12.80±0.10a	9.90±0.20f	10.60±0.15e	11.50±0.15c	10.10±0.20f	10.90±0.11d	11.80±0.05b
SOD	19.30±0.15h	21.30±0.15f	22.40±0.11e	20.50±0.10g	23.50±0.20d	24.10±0.15c	20.60±0.20g	24.50±0.36b	25.60±0.15a
POD	0.65±0.01g	0.89±0.02e	0.92±0.01e	0.69±0.01fg	1.05±0.03d	1.16±0.04c	0.72±0.01f	1.27±0.04b	1.34±0.02a
CAT	25.70±0.12f	27.70±0.19d	28.40±0.32c	26.40±0.24e	29.50±0.20b	30.50±0.26a	26.80±0.11e	29.90±0.14b	30.70±0.55a

Table 4. Effects of 1 mM ascorbic acid (AA) or 1 mM proline (Pro) applications on the thickness of midvein, blade, mesophyll, adaxial epidermis, abaxial epidermis and vessel diameter [μm] of leaves of the barley plants grown under the 100 or 200 mM NaCl stress. Means ± SD, n = 5. Mean values in each column followed by different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range test.

Parameters	0 (control)	100	200	0 + AA	100 + AA	200 + AA	0 + Pro	100 + Pro	200 + Pro
Midvein	570±15bc	430±10e	400±5.0f	610±10a	590±13ab	540±14d	590±17ab	560±8.0cd	540±6.0d
Blade	310±8.0a	170±5.0e	180±9.0e	240±5.0c	280±7.0b	270±9.0b	210±6.0d	220±6.0d	180±4.0e
mesophyll	240±5.0a	100±2.0g	130±6.0e	150±3.0d	170±7.0c	220±8.0b	130±6.0e	150±5.0d	120±3.0f
Adax. ep.	40±2.0c	40±3.0c	30±1.5d	50±1.3b	60±3.0a	30±2.1d	50±2.0b	40±1.0c	30±2.0d
Abax. ep.	30±1.0c	30±2.0c	20±0.5d	40±2.0b	50±3.0a	20±1.0d	30±1.0c	30±1.5c	30±2.0c
Vessel dia.	33±1.0c	26±2.0d	22±0.5e	33±0.2c	37±2.5a	36±0.6b	28±1.0d	32±0.8c	25±1.0

Discussion

The present study shows that the NaCl stress reduced all the studied growth traits. These inhibitory effects of NaCl stress on plant growth and biomass production are well known (Hajer *et al.* 2006, Alqurainy 2007, Long *et al.* 2008, Ma *et al*. 2013). In addition, salinity leads to dehydration and a decrease in the pressure potential (Munns 2002) and consequently to a reduction in the meristem activity and cell elongation (Shah 2007). In the present investigation, soaking the seeds in AA greatly increased the growth traits of the barley plants under the following NaCl stress as compared to the stress alone. These results are in agreement with the AA induced improvement in the salt tolerance of wheat (Athar *et al.* 2008, 2009) and sorghum (Arafa *et al.* 2009). The application of proline also increased the growth traits of the barley plants under the NaCl stress as compared to the plants grown under the stress alone. Under stress conditions, proline acts as osmoprotectant (Hartzendorf and Rolletschek 2001), membrane stabilizer (Bandurska 2001) and ROS scavenger (Matysik *et al.* 2002). The acceleration of the activities of antioxidant enzymes and an increased pool of endogenous proline found in the

present study resulted in an increase in the tolerance to the NaCl stress manifested in terms of improved growth traits and in the content of photosynthetic pigments. The reduction in growth traits in plants subjected to NaCl stress is often associated with a decrease in photosynthetic pigments, and a reduction in Chl content due to a NaCl stress was also reported in maize, wheat, canola, *etc.* (Ali *et al.* 2007). Soaking the seeds in AA or proline increased the Chl *a*, Chl *b*, and Car content in the presence or absence of the NaCl stress. Similar findings were obtained by Khan *et al.* (2010) in *Brassica campestris*. An increase in the NaCl concentration significantly reduced the total soluble sugars content of the barley leaves. This might be due to the consumption of the total soluble sugars during synthesis of antioxidants or osmoprotectants including proline. Soaking the seeds in AA or proline increased the total soluble sugars content of the barley leaves in presence or absence of the NaCl stress. It may be important as sugars also act as osmoprotectants in many plants (Everard *et al.* 1994). As mentioned above, the proline content significantly increased in the barley seedlings exposed to the salt stress and might serve as a compatible solute (Vinocur and Altman 2005). It was shown that proline accumulates in a larger amount than any other amino acids and regulates the cell osmotic potential (Ali *et al.* 1999). It is also hypothesized that proline, besides being an osmolyte, is also involved in scavenging free radicals (Okuma *et al.* 2004, Ashraf and Foolad 2007). It has been found that a salt stress up-regulated the enzymes involved in proline biosynthesis (Hare *et al.* 1999, Munns 2005). The proline content of the barley leaves also shows an increasing trend after the AA application under the NaCl stress. Similar results have been reported in sorghum (Azooz *et al.* 2004), rice (Gurmani *et al.* 2006), maize (Hussein *et al.* 2007) and tobacco (Celik and Atak 2012), where applications of various growth regulators increase the proline content of plants.

The activities of antioxidant enzymes (SOD, POD, and CAT) in the barley plants were increased under the NaCl stress as well as after the AA or proline application. These higher SOD, POD, and CAT activities help plants in defence to possible oxidative damage. Azevedo *et al.* (2011) suggest that an increase in the antioxidant enzymes helps plants maintain their growth under stress and may be regarded as indicators of salinity tolerance. Similarly to our results, Athar *et al.* (2008, 2009) reported an increase in antioxidant enzyme activities in wheat plants after an AA application.

The increased NaCl concentration markedly reduced the leaf thickness and diameter of vessels. Merkulov *et al.* (1997) observed smaller epidermal cells, thicker cuticle, and a greater number of layers of smaller mesophyll cells in leaf blade of sugar beet genotypes subjected to a drought stress. In the present study, soaking the seeds in AA or proline in the absence or presence of the NaCl stress induced an increase in the leaf thickness. AA plays multiple roles in cell division and cell wall expansion (Smirnoff and Wheeler 2000, Conklin 2001, Pignocchi and Foyer 2003). To the best of my knowledge, this is the first report about the effect of AA or proline application on the anatomical structure of the barley leaves.

In conclusion, AA or proline may be involved in an inhibition of oxidative damage in barley cells through an enhancement of enzymatic antioxidants responsible for regulation of ROS during NaCl stress. The elevated activity of antioxidant system, at least in part, protected the photosynthetic machinery and increased the tolerance of the barley plants to the NaCl stress.

References

Ali, G., Srivastava, P.S., Iqbal, M.: Proline accumulation, protein pattern and photosynthesis in regenerants grown under NaCl stress. - Biol. Plant. **42**: 89-95, 1999.

Ali, Q., Ashraf, M., Athar, H.U.R.: Exogenously applied proline at different growth stages enhances growth of two maize cultivars grown under water deficit conditions. - Pak. J. Bot. **39**: 1133-1144, 2007.

Alqurainy, F.: Responses of bean and pea to vitamin C under salinity stress. - Res. J. agr. biol. Sci. **3**: 714-722, 2007.

Association of Official Agricultural Chemists: Official Methods of Analysis. 15th Ed. - Benjamin Franklin Station, Washington 1990.

Arafa, A.A., Khafagy, M.A., El-Banna, M.F.: The effect of glycinebetaine or ascorbic acid on grain germination and leaf structure of sorghum plants grown under salinity stress. - Aust. J. Crop Sci. **3**: 294-304, 2009.

Arnon, D.I.: Copper enzymes in isolated chloroplast. Polyphenol-oxidase in *Beta vulgaris* L. - Plant Physiol. **24**: 1-5, 1949.

Ashraf, M., Athar, H.R., Harris, P.J.C., Kwon, T.R.: Some prospective strategies for improving crop salt tolerance. - Adv. Agron. **97**: 45-110, 2008.

Ashraf, M., Foolad, M.R.: Roles of glycinebetaine and proline in improving plant abiotic stress tolerance. - Environ. exp. Bot. **59**: 206-216, 2007.

Ashraf, M., Rahmatullah, R., Ahmad, R., Bhatti, A.S., Afzal, M., Sarwar, A. Maqsood, M. A. Kanwal, S.: Amelioration of salt stress in sugarcane (*Saccharum officinarum* L.) by supplying potassium and silicon in hydroponics. - Pedosphere **20**:153-162, 2010.

Athar, H.R., Khan, A., Ashraf, M.: Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat. - Environ. exp. Bot. **63**: 224-231, 2008.

Athar, H.R., Khan, A., Ashraf, M.: Inducing salt tolerance in wheat by exogenously applied ascorbic acid through different modes. - J. Plant Nutr. **32**: 1799-1817, 2009.

Azevedo, R.A., Carvalho, R.F., Cia, M.C., Gratão, P.L.: Sugarcane under pressure: an overview of biochemical and physiological studies of abiotic stress. - Trop. Plant Biol. **4**: 42-51, 2011.

Azooz, M.M.: Proteins, sugars and ion leakage as a selection criterion for the salt tolerance of three sorghum cultivars at seedling stage grown under NaCl and nicotinamide. - Int. J. agr. Biol. **6**: 27-35, 2004.

Azooz, M.M., Shaddad, M.A., Abdel Latef, A.A.: The accumulation and compartmentation of proline in relation to salt tolerance of three sorghum cultivars. - Indian J. Plant Physiol. **9**: 1-8, 2004.

Bandurska, H.: Does proline accumulated in the leaves of water deficit stressed barley plants confine cell membrane injuries? II. Proline accumulation during hardening and its involvement in reducing membrane injuries in leaves subjected to severe osmotic stress. - Acta Physiol. Plant. **23**: 483-490, 2001.

Bassuony, F.M., Hassanein, R.A., Baraka, D.M., Khalil, R.R.: Physiological effects of nicotinamide and ascorbic acid on *Zea mays* plant grown under salinity stress. II. Changes in nitrogen constituent, protein profiles, protease enzyme and certain inorganic cations. - Aust. J. appl. Sci. **2**: 350-359, 2008.

Bates, L.S., Waldren, R.P.,Teare, I.D.: Rapid determination of free proline for water stress studies. - Plant Soil **39**: 205-207, 1973.

Beltagi, M.S.: Exogenous ascorbic acid (vitamin C) induced anabolic changes for salt tolerance in chick pea (*Cicer arietinum* L.) plants. - Afr. J. Plant Sci. **2**: 118-123, 2008.

Bohnert, H.J., Jensen, R.G.: Strategies for engineering water-stress tolerance in plants. - Trends Biotechnol. **14**: 89-97, 1996.

Celik, O., Atak, C.: The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties. - Turk. J. Biol. **36**: 327-338, 2012.

Conklin, P.L.: Recent advances in the role and biosynthesis of ascorbic acid in plants. - Plant Cell Environ. **24**: 383-394, 2001.

Conklin, P.L., Barth, C.: Ascorbic acid: a familiar small molecule intertwined in the response of plants to ozone, pathogens and the onset of senescence. - Plant Cell Environ. **27**: 959-970, 2004.

Dionisio-Sese, M.L., Tobita, S.: Antioxidant responses of rice seedlings to salinity stress. - Plant Sci. **135**: 1-9, 1998.

Dolatabadian, A., Saleh, R.J.: Impact of exogenous ascorbic acid on antioxidant activity and some physiological traits of common bean subjected to salinity stress. - Notulae bot. agrobot. Cluj-Napoca **37**: 165-172, 2009.

Everard, J.D., Gucci, R., Kann, S.C., Flore, J.A., Loescher, W. H.: Gas exchange and carbon partitioning in the leaves of celery (*Apium graveolens* L) at various levels of root zone salinity. - Plant Physiol. **106**: 281-292, 1994.

Ferreira-Silva, S.L., Voigt, E.L., Silva, E.N., Maia, J.M., Aragao, T.C.R.: Partial oxidative protection by enzymatic and non-enzymatic components in cashew leaves under high salinity. - Biol. Plant. **56**: 172-176, 2012.

Fridovich, I.: Superoxide dismutase. - Annu. Rev. Biochem. **44**: 147-159, 1975.

Gurmani, A.R., Bano, A., Salim, M.: Effect of growth regulators on growth, yield and ion accumulation of rice (*Oryza sativa* L.) under salt stress. - Pak. J. Bot. **38**: 1415-1424, 2006.

Hajer, A.S., Malibari, A.A., Al-Zahrani, H.S., Almaghrabi, O.A.: Responses of three tomato cultivars to sea water salinity 1. Effect of salinity on the seedling growth. - Afr. J. Biotechnol. **5**: 855-861, 2006.

Hamdia, M.A., Shaddad, M.A.K.: Salt tolerance of crop plants. - J. Stress Physiol. Biochem. **6**: 64-90, 2010.

Hare, P.D., Cress, W.A., Van Staden, J.: Proline synthesis and degradation: a model system for elucidating stress related signal transduction. - J. exp. Bot. **50**:413-434, 1999.

Hartzendorf, T., Rolletschek, H.: Effect of NaCl-salinity on amino acid and carbohydrate contents of *Phragmites australis*. - Aquat. Bot. **69**: 195-208, 2001.

Hoque, M.A., Okuma, E., Banau, M.N.A., Nakamura, Y., Shimoishi, Y., Murata, N.: Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betain by increasing antioxidants enzyme activity. - J. Plant Physiol. **64**: 553-561, 2007.

Hussain, G., Al-Jaloud, A.A., Al-Shammary, A.A., Karimulla, S., Al-Asward, S.O.: Effect of saline irrigation on germination and growth parameters of barley (*Hordeum vulgare* L.) in a pot experiment. - Agr. Water Manage. **34**:125-135, 1997.

Hussein, M.M., Balbaa, L.K., Gaballah, M.S.: Salicylic acid and salinity effects on growth of maize plants. - Res. J. agr. biol. Sci. **3**: 321-328, 2007.

Kaya, C., Tuna, A.L., Dikilitas, M. Cullu, M. A.: Responses of some enzymes and key growth parameters of salt stressed maize plants to foliar and seed applications of kinetin and indole acetic acid. - J. Plant Nutr. **33**: 405-422, 2010.

Khan, A., Ahmad, M.S.A., Athar, H.R., Ashraf, M.: Interactive effect of foliarly applied ascorbic acid and salt stress on wheat (*Triticum aestivum* L.) at the seedling stage. - Pak. J. Bot. **38**:1407-1414, 2006.

Khan, A., Iqbal, I., Shah, A., Ahmad, A., Ibrahim, M.: Alleviation of adverse effects of salt stress in brassica (*Brassica campestris*) by pre-sowing seed treatment with ascorbic acid. - J. Agr. environ. Sci. **7**: 557-560, 2010.

Khosravinejad, H.F.R., Farboondia, T.: Effect of salinity on photosynthetic pigments, respiration and water content in barley varieties. - Pak. J. biol. Sci. **11**: 2438-2442, 2008.

Long, X.H., Mehta, S.K., Liu, Z.P.: Effect of NO-3-N enrichment on seawater stress tolerance of Jerusalem artichoke (*Helianthus tuberosus*). - Pedosphere **19**: 113-123, 2008.

Luck, H.: Catalase estimation. - In: Bergmeyer, H.U. (ed.): Methods of Enzymatic Analysis. Vol. 2. Pp. 885-888. Academic Press, New York 1975.

Ma, L.J., Yu, C.M., Li, X.M., Li, Y.Y., Wang, L.L., Ma, C.Y., Tao, S.Y., Bu, N.: Pretreatment with NaCl induces tolerance of rice seedlings to subsequent Cd or Cd + NaCl stresses. - Biol. Plant. **57**: 567-570, 2013.

Makela, P., Kontturib, M., Pehua, E., Somersaloa, S.: Photosynthetic response to drought and salt stressed tomato and turnip rape plants to foliar applied glycinebetaine. - Physiol. Plant. **105**: 45-50, 1999.

Matysik, J., Aliab, B., Mohanty, P.: Molecular mechanism of quenching of reactive oxygen species by proline under stress in plants. - Curr. Sci. **82**: 525-532, 2002.

Mer, R.K., Prajith, P.K., Pandya, D.H., Pandey, A.N.: Effect of salts on germination of seeds and growth of young plants of *Hordeum vulgare*, *Triticum aestivum*, *Cicer arietinum* and *Brassica juncea*. - J. Agron. Crop Sci. **185**: 209-216, 2000.

Merkulov, L.J., Ivezić,J., Krstić, B., Kovačev, L., Pajević, S., Vujičić, D: Structural characteristics of leaf blade of differentially drought-tolerant sugar beet genotypes. - In: Vujičić, D. (ed.): Proceedings of the International Symposium: Drought and Plant Production. Pp. 487-492. Agricultural Research Institute "Serbia", Belgrade 1997.

Munns, R.: Comparative physiology of salt and water stress. - Plant Cell Environ. **25**: 239-250, 2002.

Munns, R.: Genes and salt tolerance: bringing them together. - New Phytol. **167**: 645-663, 2005.

Nawaz, K., Hussain, K., Majeed, A., Khan, F., Afghan, S., Ali, K.: Fatality of salt stress to plants: morphological, physiological and biochemical aspects. - Afr. J. Biotechnol.

9: 5475-5480, 2010.

Okuma, E., Murakami, Y., Shimoishi, Y., Tada, M., Murata, Y.: Effects of exogenous application of proline and betaine on the growth of tobacco cultured cells under saline conditions. - Soil Plant Nutr. **50**: 1301-1305, 2004.

Pignocchi, C., Foyer, C.: Apoplastic ascorbate metabolism and its role in the regulation of cell signaling. - Curr. Opin. Plant Biol. **6**: 379-389, 2003.

Rahman, S.M.D., Miyake, H., Takeoka, Y.: Effects of exogenous glycinebetaine on growth and ultrastructure of salt stressed rice seedlings (*Oryza sativa* L.) - Plant Prod. Sci. **5**: 33-44, 2002.

Raza, S.H., Athar, H.U.R., Ashraf, M.: Influence of exogenously applied glycinebetaine on the photosynthetic capacity of two differently adapted wheat cultivars under salt stress. - Pak. J. Bot. **38**: 341-351, 2006.

Rhodes, D., Hanson, A.D.: Quaternary ammonium and tertiary sulfonium compounds in higher plants. - Annu. Rev. Plant Physiol. Plant mol. Biol. **44**: 357-384, 1993.

Roy, D., Basu, N., Bhunia, A., Banerjee, S.K.: Counteraction of exogenous L-proline with NaCl in salt-sensitive cultivar of rice. - Biol. Plant. **35**: 69-72, 1993.

Sass, J.A.: Botanical Microtechnique. 3rd Ed. - The Iowa State University Press, Ames 1961.

Serraj, R., Sinclair, T.R.: Osmolyte accumulation: can it really help increase crop yield under drought conditions? - Plant Cell Environ. **25**: 333-341, 2002.

Shah, S.H.: Effects of salt stress on mustard as affected by gibberellic acid application. - Genet. Appl. Plant Physiol. **3391**: 97-106, 2007.

Smirnoff, N., Wheeler, G.L.: Ascorbic acid in plants: biosynthesis and function. - Crit. Rev. Biochem. mol. Biol. **35**: 291-314, 2000.

Thomas, R.L., Jen, J.J., Morr, C.V.: Changes in soluble and bound peroxidase, IAA oxidase during tomato fruit development. - J. Food Sci. **47**: 158-161, 1981.

Tuna, A.L., Kaya, C., Dikilitas, M., Higgs, D.E.B.: The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. - Environ. exp. Bot. **62**: 1-9, 2008.

Vinocur, B., Altman, A.: Recent advances in engineering plant tolerance to aboitic stress: achievements and limitations. - Curr. Opin. Biotechnol. **16**:123-132, 2005.

Yamasaki, S., Dillenburg, L.R.: Measurements of leaf relative water content in *Araucaria angustifolia* 1. - Rev. brasil. Fisiol. veg. **11**: 69-75, 1999.

Yancey, P.H., Clark, M.B., Hands, S.C., Bowlus, R.D., Somero, G.N.: Living with water stress: evaluation of osmolyte systems. - Science **217**: 1214-1222, 1982.

Zhen, A., Bie, Z.L., Huang, Y., Liu, Z.X., Fan, M.L.: Effects of 5-aminolevulinic acid on the H_2O_2-content and antioxidative enzyme gene expression in NaCl-treated cucumber seedlings. - Biol. Plant. **56**: 566-570, 2012.