Response of potato species to salt and osmotic stress in vitro and the role of acetylsalicylic acid: non-enzymatic antioxidants

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Abstract

In a series of experiment, the response of two contrasting potato species, namely, *Solanum acaule* (tolerant) and *Solanum tuberosum* cv. Agria (intolerant) to salt (80 mM NaCl) and drought stress (15% polyethylene glycol; PEG) was studied *in vitro.* Furthermore, the role of acetyl salicylic acid (ASA) (1 and 10 µM) in alleviating oxidative stress was investigated. In Agria cultivar, NaCl and PEG reduced shoot dry weight, carotenoids, phenolics, anthocyanins, flavonoids, ascorbate and glutathione pool, proline dehydrogenase (ProDH) and phenylalanine ammonia-lyase (PAL) activity. NaCl and PEG also increased proline content, malondealdehyde (MDA) level and the activity of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and the effect of PEG was more severe. In *S. acaule*, on the contrary, NaCl had no effect on shoot dry weight and MDA, but increased the contents of carotenoids, phenolics, anthocyanins**,** flavonoids, ascorbate and glutathione pool as well as the activity of P5CS and PAL and proline content**.** However, the response of this species to PEG was different from that of NaCl and reductions in shoot dry weight and in most antioxidants and an increase in MDA were observed. ASA, especially 1 µM, profoundly improved plant performance in both species and under both types of stress via increasing the PAL and P5CS activity and most non-enzymatic antioxidants contents and decreasing ProDH activity.

Keywords: acetylsalicylic acid, non-enzymatic antioxidant systems, oxidative stress, osmotic stress, salt s tress

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Introduction

Plants are frequently exposed to diverse stress conditions, including salt, drought, heat, low temperature, heavy metals, and oxidative stress. Nowadays, approximately 20% of the world's irrigated lands are salt-affected (Ashraf and Foolad, 2007) which is one of the principal limiting factors in crop productivity. Salt induces several types of plant changes, including altered nutrient uptake, the accumulation of toxic ions, especially Na^+ and osmotic and oxidative stress. Drastic changes in ion and water homeostasis induced by salinity result in molecular damage,

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growth arrest, and even cell death (Sudhakar et al., 2001).

Drought stress is also a salient factor in reducing the yield of major crops and continuous reduction in water resources has been identified as a serious threat to sustainable food production. Different plants use different strategies to counteract osmotic stresses (salinity and drought). For example, mechanism of stress tolerance has been reported to be associated with hyper-accumulation of compatible solutes such as proline, glycine betain and soluble carbohydrates (Ashraf and Foolad, 2007; Rahnama and Ebrahimzadeh, 2004; Verbuggen and Hermans, 2008). It is now well evident that these solutes contribute to prevent dehydration and cellular damage by osmotic adjustment (Ashraf and Foolad, 2007; Parida and Das, 2005). Abiotic stress conditions such as salinity and drought favor the accumulation of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide (Parida and Das, 2005; Sudhakar et al., 2001). ROS interact with a wide range of molecules causing pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and DNA mutation (Molassiotis et al*.,* 2006). Malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids, has been utilized as a biomarker of ROS (Iturbe-Ormaexte et al*.*, 1998; Juan et al*.*, 2005). Antioxidant defense systems in plants include both enzymatic antioxidants such as superoxide dismutase, catalase, ascorbate peroxidase, peroxidase and glutathione reductase, and non-enzymatic antioxidants such as ascorbate, glutathione, carotenoids and some phenolic compounds (Juan et al*.*, 2005; Parida and Das, 2005; Solecka, 1997; Sudhakar et al., 2001; Syvacy and Sokmen, 2004).

The experimental environment for salinity and drought studies should be consistent, highly controlled and monitored since fluctuating environmental conditions confound the exact plant response to salt and water stresses. *In vitro* cultures can provide an effective alternative to avoid soil or environmental complexities when studying plant response to imposed stress factors, and can offer a means to focus exclusively on physiological and biochemical processes which contribute to salinity and drought tolerance (Shibli et al*.*, 2007; Aqeel Ahmad et al*.*, 2007).

One of the factors that complicates the study of salt stress is the fact that salt is both an ionic and an osmotic component (Silva et al*.*, 2008). The use of both an osmotic agent and salt can help to discern specific ion toxicity effects from those induced by the osmotic component. Polyethylene glycol (PEG) is the most widely used osmoticum to study the water status of plants. On the other hand, NaCl is an ionic and penetrating stress agent, as it is well known to produce specific ionic toxicities in plant culture studies. In addition, it can be easily taken up by the cultured cells and can cause ionic as well as osmotic effects (Aqeel Ahmad et al*.*, 2007).

The alleviation of oxidative damage and increased resistance to environmental stresses are often correlated with an efficient antioxidative system. Such systems may be induced or enhanced by the application of chemicals such as salicylic acid (SA) or its derivates. SA is considered to be a hormone-like substance that is important in the regulation of plant growth and development. Ion uptake and transport, photosynthetic rate, stomatal conductance and transpiration may all be affected by SA application. It is one of the suitable endogenous hormones that regulate the synthesis of antioxidant enzymes during abiotic and biotic stresses. This role suggests that it could alleviate salt and drought stresses in plants, and that it may function in this manner if applied exogenously (Horvath et al*,.* 2007 a, b; Raskin, 1992; Senaratna et al*.,* 2000).

In the present study, we aimed to investigate the non-enzymatic mechanisms involved in salt and drought tolerance in two contrasting potato species (Arvin and Donnelly, 2008) and the role of ASA in nullifying the adverse effect of stresses *in vitro*.

Materials and Methods

Plant materials and treatments

True potato seeds from wild species *Solanum acaule* (3760) (accession in brackets) were received from the United States Department of Agriculture Research Service, Inter-Regional Potato Introduction Station, at

Stargeon Bay, WI., USA and held at fridge temperature (4 °C) until required. Seeds were disinfested for 20 min in 30% household bleach and 70% ethanol for 1 min, rinsed 3 times with sterile distilled water, soaked for 24 h in 2 g/l filter-sterilized gibberellic acid (GA_3) solution to break dormancy, again rinsed with sterile distilled water, and aseptically transferred to 0.8 % agar plates for *in vitro* germination.

The plants (wild species: *S. acaule* and cultivar: Agria) were maintained by subculture of nodal cuttings on sterile liquid medium consisting of MS (Murashige and Skoog, 1962) salts and vitamins and 3% sucrose without agar (pH 5.7) in phytacone vessels (Sigma, USA). The cultures were incubated at 25±2 °C with 16/8 h D/N at 40 µmol m^{-2} s^{-1} photon flux density (cool white fluorescent light).

NaCl (80 mM), PEG (15%) and ASA (1 and 10 µM) were added to the medium before being autoclaved. The water potentials of media, determined by a vapor pressure osmometer (VAPRO-5520, Wescure Inc., Logan, Utah, USA), were -0.4 MPa for 15% PEG (6000) and 80 mM NaCl (iso-osmotic), and -0.17 MPa for control (MS medium). After adding iso-osmotic agents to MS medium, the final water potential of media was -0.57 MPa. For ASA pretreatments, three single nodes of plants were cultured in phytacone vessels, including liquid MS medium and/or liquid MS medium + ASA. After one week, these explants were aseptically transferred to liquid MS medium, with or without iso-osmotic agents (15% PEG and 80 mM NaCl). The culture conditions were the same as above. After 4 weeks, morphological parameters were recorded for each treatment and the plant materials were frozen in liquid nitrogen and stored at -80 °C for subsequent analysis (all experiments were done on the shoot samples).

Growth parameters

Shoot dry weight (g/plant) was determined after the samples were oven dried at 70 °C for 48 h and then weighed.

Mineral content

Shoot Na^+ content was determined using an atomic absorption spectrophotometer (Shimadzu, model 610, Japan) after wet digestion of the ash with nitric acid and expressed as mg g^{-1}d w.

Lipid peroxidation

The level of lipid peroxidation in plant tissues was measured by determination of malondialdehyde (MDA) which is known to be the breakdown product of lipid peroxidation (Heath and Packer, 1969). The shoot samples (0.1 g) were homogenized in 10 ml of 0.1% trichloroacetic acid (TCA), then centrifuged at 10000×g for 15 min. 1 ml of supernatant was then vortexed with 4 ml of 20% TCA containing 0.5% 2-thiobarbituric acid (TBA), and the solution was heated for 30 min at 90 °C. The samples were cooled on ice for 5 min and re centrifuged for 10 min at 10000×g. Non-specific absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm for MDA measurement. For MDA calculation, an extinction coefficient (ε) of 1.55×10^5 $M^{-1}Cm^{-1}$ was used at 532 nm. Results were expressed as nmol g^{-1} f w.

Carotenoids

Shoot samples (0.25 g) were homogenized in acetone (80 %) then the extract was centrifuged at 3000×g and the amount of carotenoids was determined according to the method of Lichtenthaler (1987). Content of carotenoids was expressed as µg g^{-1} f w.

Total phenolic content

Phenolic compounds were determined using the Soland and Laima (1999) method. Phenolics of plant materials (shoot samples) were extracted with 95% ethanol. The extract was mixed with 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1 with water) then 1 ml of a 5% sodium carbonate solution was also added. The absorption at 725 nm was measured after 1 h. Gallic acid was used as a standard and results were expressed as mg g^{-1} f w.

Anthocyanins

To determine anthocyanins content by the method of Wanger (1979), frozen tissue samples (0.1 g) were soaked in 10 ml acidified methanol (methanol: HCl 99:1 (V/V)). Tissues were crushed and kept at 25 °C for 24 h in the dark. The extract was then centrifuged at 4000×g for 5 min at room temperature, and the absorption at 550 nm of the supernatant was read by the spectrophotometer. To calculate the anthocyanins content, the extinction coefficient 33000 mol^{-1} cm^{-1} was used and anthocyanins content was expressed as µmol g^{-1} fw.

Flavonoids

The flavonoids content was determined as described by Krizek et al. (1998). Shoot samples (0.1 g) were extracted with 10 ml of acidified ethanol (ethanol: acetic acid, 99: 1 (V/V)). The samples were gently boiled for 10 min in water bath at 80 °C and the absorbance was measured at three wavelengths: 270, 300 and 330 nm. Results were expressed as percentage of absorbance (% A).

Ascorbate

Ascorbate (ASA), dehydroascorbate (DHAs) and total ascorbate were determined as described by De Pinto et al. (1999). Shoot sample (0.5 g) was homogenized in 10 ml meta phosphoric acid 5% and centrifuged for 15 min at 1000×g. 300 µl of supernatant was used for the ASA assay and these solutions (750 µl potassium phosphate (100 mM, pH 7.2) and 300 µl distilled water) were added to the extract, respectively. 300 µl of supernatant was used for the total ascorbate assay and the following solutions were added 750 µl potassium phosphate (100 mM, pH 7.2) and 150 µl of 10 mM dithiotheritol. The samples were incubated at room temperature for 10 min, and then 150 µl of 0.5% N-ethylmaleimide was added. Both samples were vortexed and incubated at room temperature for 10 min. 600 µl of 10% (W/V) TCA, 600 µl of 44% (V/V) ortho phosphoric acid, 600 µl of 4% (W/V) bipyridyl in 70% (V/V) ethanol and 10 µl of 3% $FeCl_3$ were added to each sample. After vortex-mixing, the samples were incubated at 40 °C in a water bath for 20 min, and then were vortexed again and incubated at 40 °C water bath for another 20 min. The absorbance of samples at 525 nm was recorded. A standard curve of ASA was used for the calculation of ASA and total ASA concentration. ASA, DHAs and total ascorbate (ASA + DHAs) concentrations were expressed as mg g^{-1} f w.

Glutathione

The levels of glutathione were measured spectrophotometrically by monitoring the reduction of 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, after the method of Griffith (1980). Shoot sample (0.5 g) was homogenized in 10 ml meta phosphoric acid 5% and centrifuged for 15 min at 1000×g. 300 µl of supernatant was used for the glutathione assay. For measurements of total glutathione (GSH + GSSG), 150 µl of neutralized extract were added to 1.2 ml of 0.3 mM NADPH, 150 µl of 6 mM DTNB and 1 unit of glutathione reductase (GR). For oxidized glutathione (GSSG) determination, 150 µl of neutralized extract were incubated with 2 µl of 2-vinylpyridine for 1 h at 25 °C, and then added to 1.2 ml of 0.3 mM NADPH, 100 µl of 6 mM DTNB, and 1 unit of GR. The concentration of reduced glutathione (GSH) was calculated as the difference between total glutathione and GSSG. A standard curve was used for the calculation of glutathione concentration. GSH, GSSG and total glutathione (GSH + GSSG) concentration were expressed as mg g^{-1} f w.

Proline

Free proline was determined according to Bates et al. (1973). Shoot samples (0.5 g) from each group were homogenized in 3% (W/V) sulphosalicylic acid and then the homogenate was centrifuged. The mixture was heated at 100 °C for 1 h in water bath after addition of acid ninhydrin and glacial acetic acid. Reaction was then stopped by ice bath. The mixture was extracted with toluene and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was

determined using calibration curve and expressed as mg g^{-1}f w.

Enzyme extractions and assays

Frozen shoot samples (0.5 g) were homogenized in 2.5 ml of 50 mM phosphate buffer (pH 7.2) containing 1 mM ethylendiamine tetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1% polyvinyl pyrrolidone (PVP). The homogenate solution was centrifuged at 14000×g for 15 min at 4 °C and the clear supernatant was used directly for the assay of enzymes activity (Δ^1-pyrroline-5-carboxylate synthetase and proline dehydrogenase) and estimation of protein. The activity of enzymes was determined at 25 °C with a spectrophotometer (Cary 50, Germany). The supernatant was used to measure total soluble protein according to Bradford (1976) and expressed in mg g^{-1} f w. Bovine serum albumin was used as standard (data not shown).

Phenylalanine ammonia-lyase (PAL) (EC 4. 3. 1. 5)

PAL was extracted from shoot samples (0.3 g) with 6.5 ml of 50 mM Tris-HCl buffer (pH 8.8) containing 15 mM β-mercaptoethanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged at 50000×g for 30 min, and the supernatant was collected for enzyme assay.

PAL activity was determined based on the rate of cinnamic acid production as described by Wang et al. (2006). Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of double distilled water and 0.1 ml of enzyme extract were incubated at 37 °C for 1 h. The reaction was terminated by the addition of 0.5 ml of 6 M HCl, and the product was extracted with 15 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 ml NaOH and the cinnamic acid concentration was quantified with the absorbance measured at 290 nm. One unit of PAL activity is equal to 1 µmol of cinnamic acid produced per min.

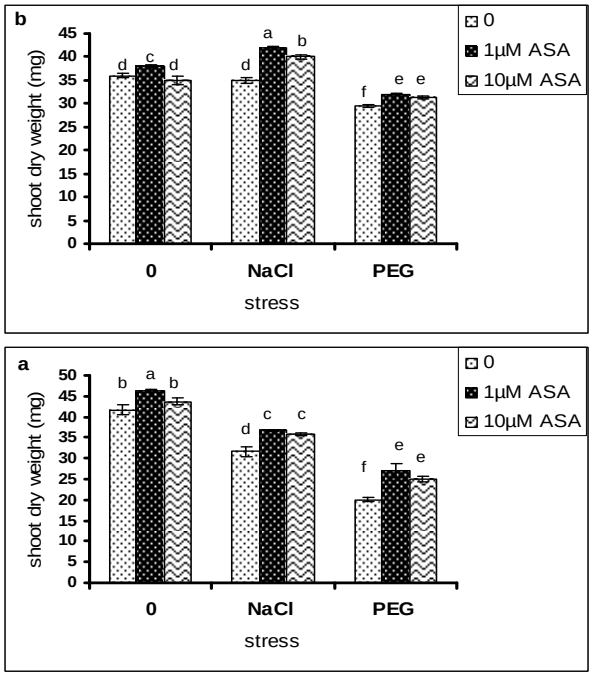

Fig. I. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot dry weight in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters show no difference ($p \leq 0.05$) according to Duncan test.

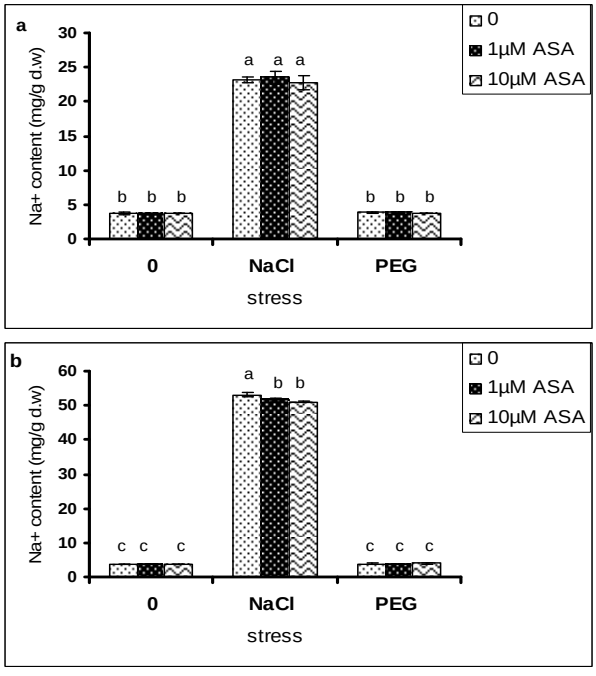

Fig. II. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot Na^+ content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters show no difference ($p \leq 0.05$) according to Duncan test.

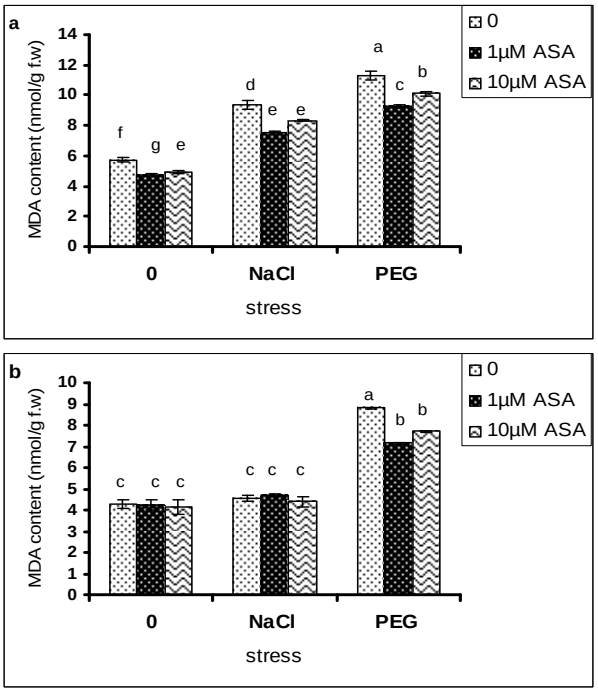

Fig. III. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot malondialdehyde content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

Δ^1-pyrroline-5-carboxylate synthetase (P5CS) (EC 1. 5. 1. 12)

The P5CS activity was determined as described by Rahnama and Ebrahimzadeh (2004). The P5CS activity was determined by monitoring the consumption of NADPH through measuring the increase in absorbance at 340 nm, in a reaction mixture containing 75 mM glutamic acid, 75 mM Tris-HCl (pH 7.3), 18.75 mM $MgCl_2$, 5 mM ATP, 0.4 mM NADPH, and 50 µl extracted protein. P5CS is expressed as unit per mg protein. One unit is defined as 0.01 increases in A_{340} per min.

Proline dehydrogenase (ProDH) (EC1.5.99. 8)

The ProDH activity was determined as described by Rahnama and Ebrahimzadeh (2004). The ProDH activity was measured by detecting the reduction of A_{340} in a reaction mixture containing 100 mM acetate buffer (pH 10.3), 20 mM L-proline, 10 mM NAD^+ and 100 µl extracted protein. ProDH is expressed as unit per mg protein. One unit is defined as 0.01 decreases in A_{340} per min.

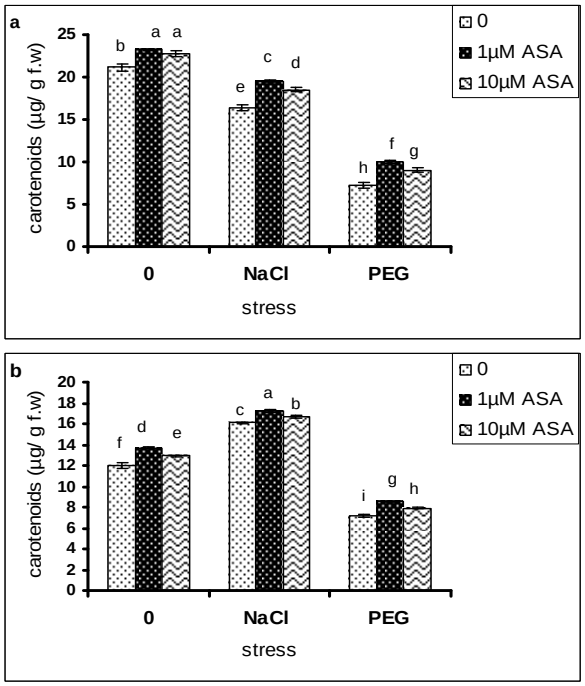

Fig. IV The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot carotenoids content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

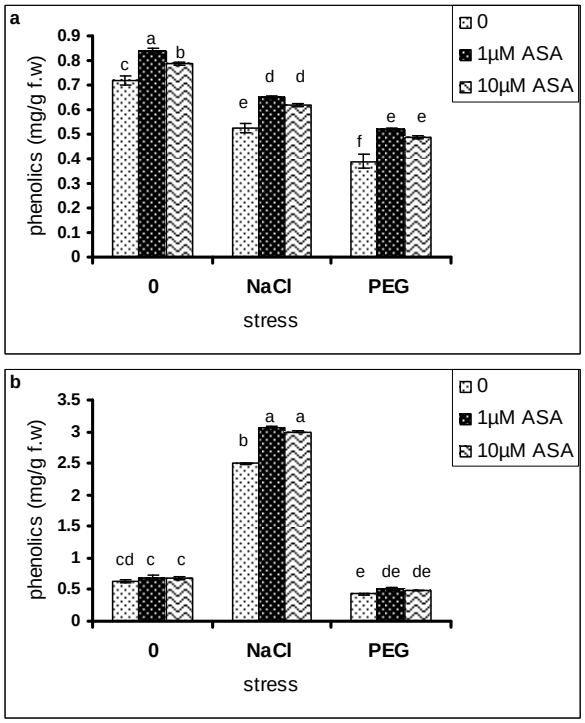

Fig. V. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot phenolics content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

Statistical analysis

The experimental design was a completely randomized design with 3 replicates of 9 treatments and repeated twice. The treatments consisted of three levels of stress (0, 80 mM NaCl and 15% PEG-6000) and three levels of ASA (0, 1 and 10 µM). The data were subjected to an analysis of variance and the statistical significance of the results was analyzed by the Duncan test ($p \leq 0.05$).

Results

Agria cultivar

Compared with the control treatment (no salt), NaCl inhibited shoot dry weight (Fig. 1a) and enhanced shoot Na^+ content (Fig. 2a) and MDA (Fig. IIIa). A significant reduction in carotenoids (Fig. IVa), phenolics (Fig. Va), anthocyanins (Fig. VIa), flavonoids (Fig. VIIa, VIIIa, IXa), total ascorbates (Fig. XIIa), reduced glutathione (Fig. XIIIa), oxidized glutathione (Fig. XIVa) and total glutathione content (Fig. XVa) was also observed. An increase in proline content (Fig. XVIa) and P5CS activity (Fig. XVIIa) and decreases in the activity of ProDH (Fig. XVIIIa) and PAL (Fig. XIXa) were also recorded in plants exposed to NaCl. However, compared with the salt stress, the effect of osmotic stress was more severe for all traits but shoot Na^+ content.

Relative to the control, ASA pretreatment increased shoot dry weight (Fig. Ia), reduced MDA (Fig. IIIa) and the activity of ProDH (Fig. XVIIIa) with no effect on shoot Na^+ contents (Fig. IIa) of plants under stress and non stress conditions. Moreover, Carotenoids (Fig. IVa), phenolics (Fig. Va), anthocyanins (Fig. VIa), dehydroascorbate (Fig. XIa), total ascorbate (Fig. XIIa), reduced glutathione (Fig. XIIIa), total glutathione content (Fig. XVa), proline (Fig. XVIa) and the activity of P5CS (Fig. XVIIa) and PAL (Fig. XIXa) were enhanced following ASA treatment under stress and non- stress conditions.

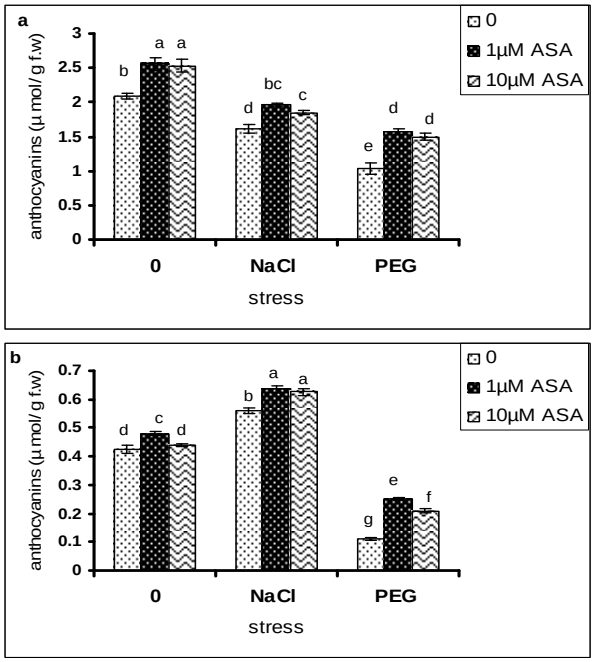

Fig. VI. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot anthocyanins content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

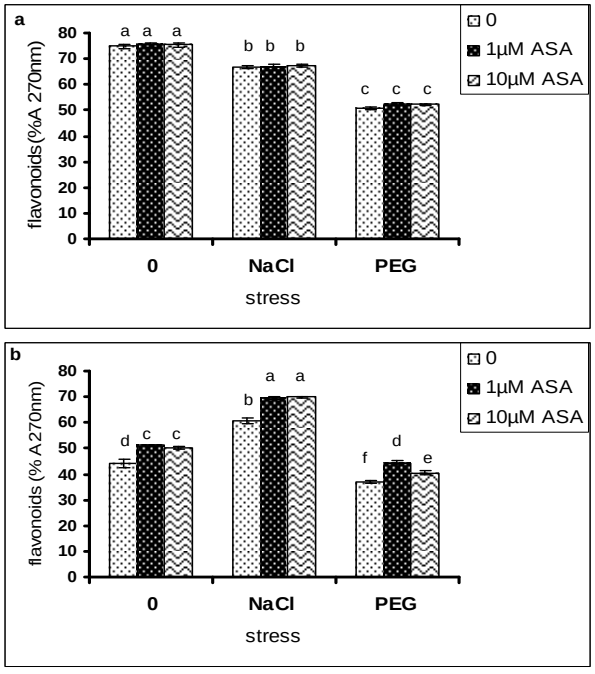

Fig. VII. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot flavonoids (%A 270nm) in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

S. acaule

Salinity had no adverse effect on shoot dry weight (Fig. Ib), but enhanced shoot Na^+ uptake (Fig. IIb). Also no significant differences were found in MDA level (Fig. IIIb), total ascorbate (Fig. XIIb) and total glutathione (Fig. XVb) in salt-stressed plants, relative to the control.

Moreover, salinity increased the content of carotenoids (Fig. IVb), phenolics (Fig. Va), anthocyanins (Fig. VIa), flavonoids (Fig. VIIb, VIIIb, IXb), dehydroascorbate (Fig. XIb), reduced glutathione (Fig. XIIIb), proline (Fig. XVIb) and also enhanced the activity of P5CS (Fig. XVIIb) and PAL (Fig. XIXb), while the ProDH activity was decreased (Fig.XVIIIb). However, the response of this species to osmotic stress was different from the salt and relative to the control.

PEG significantly reduced shoot dry weight (Fig. Ib), carotenoids (Fig. IVb), phenolics (Fig. Vb), anthocyanins (Fig. VIb), flavonoids (Fig. VIIb, VIIIb, IXb), ascorbate (Fig. Xb) and total ascorbate (Fig. XIIb), oxidized glutathione (Fig. XIVb), total glutathione content (Fig. XVb), ProDH (Fig. XVIIIb) and PAL activity (Fig. XIXb), while the content of dehydroascorbate (Fig. IIb), reduced glutathione (Fig. XIIIb), proline (Fig. XVIb), the activity of P5CS enzyme (Fig. XVIIb) and MDA (Fig. IIIb) were increased.

Compared to the control (no ASA), ASA increased shoot dry weight (Fig. Ib), the content of carotenoids (Fig. IVb), phenolics (Fig. Vb), anthocyanins (Fig. VIb), flavonoids (270nm and 330nm) (Fig. VIIb, IXb), ascorbate (Fig. Xb), dehydroascorbate (Fig. XIb), total ascorbate (Fig. XII), reduced glutathione (Fig. XIIIb), oxidized glutathione (Fig. XIVb), total glutathione (Fig. XVb) and proline (Fig. XVIb) and the activity of P5CS (Fig. XVII) and PAL (Fig. XIX) under stress conditions. Moreover, ASA reduced the activity of ProDH (Fig. XVIIIb) and had no effect on shoot Na^+ in plants subjected to NaCl or PEG (Fig. IIb). However, ASA had no effect on MDA in plants exposed to NaCl, but reduced it under osmotic stress (Fig. IIIb).

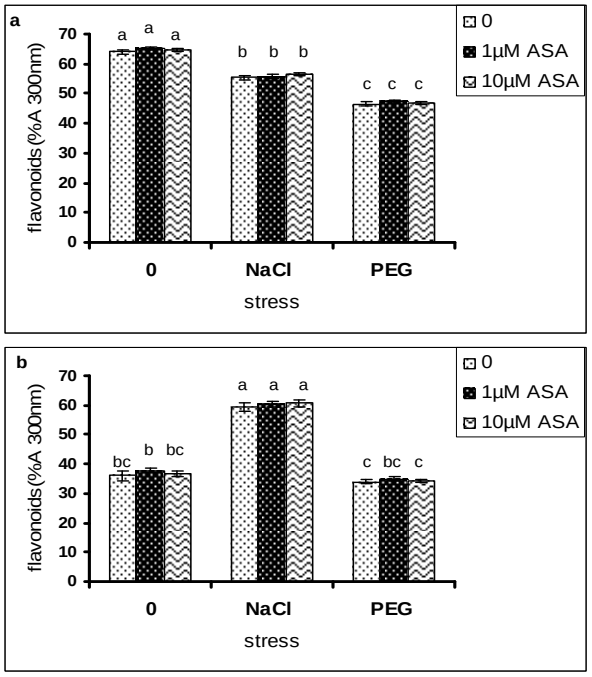

Fig. VIII. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot flavonoids (%A 300nm) in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

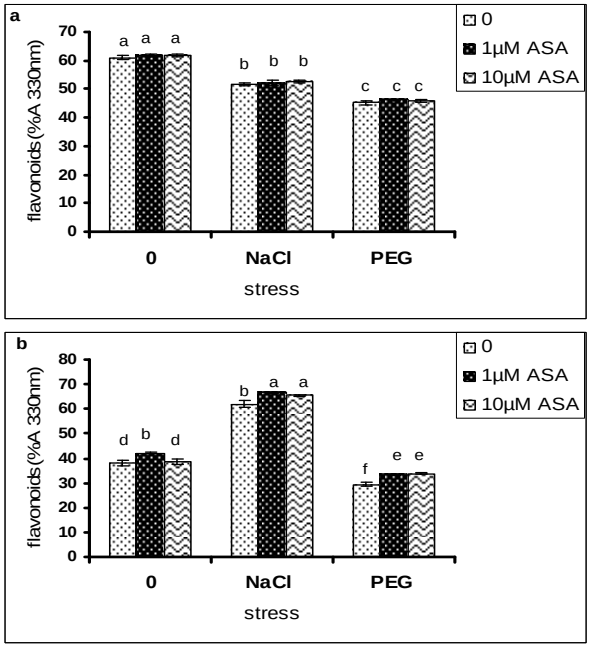

Fig. IX. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot flavonoids (%A 330nm) in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

Discussion

Under oxidative stress conditions such as salinity, drought and low or high temperature, and plants produce reactive oxygen species (ROS), which are harmful to plant growth due to their detrimental effects on the subcellular components and plant metabolism of, leading to the oxidative destruction of cells. Active oxygen species deteriorate membrane lipids and lipid peroxidation, leading to increased leakage of solutes from membranes (Gunes et al., 2007). Under non-stress conditions, ROS are efficiently eliminated by non-enzymatic and enzymatic antioxidants, whereas during saline and drought conditions the production of ROS exceeds the capacity of the antioxidative systems to remove them, causing oxidative stress (Sofo et al., 2005).

Non-enzymatic antioxidants including carotenoids, phenolics, anthocyanins, flavonoids, ascorbate and glutathione have a vital role in alleviating stress damage in plants via different mechanism(s). Carotenoids are responsible for quenching singlet oxygen and thereby avoiding lipid peroxidation and consequent oxidative damage (Juan et al., 2005). Phenolic compounds, anthocyanins and flavonoids are involved in retarding or inhibiting lipid autoxidation by acting as radical scavengers and are essential antioxidants protecting the cells against propagation of the oxidative chain (Ksouri et al., 2007; Navarro et al., 2006). Flavonoids are also powerful antioxidants that can detoxify H_2O_2 with specific peroxidase in vacuole or cell wall of plant (Yamasaki et al., 1997).

The non-enzymatic antioxidant system also includes ascorbate and glutathione, two constituents of the antioxidative ascorbate-glutathione cycle that detoxify H_2O_2 in the chloroplasts and are located both within the cell and in the apoplast (Sofo et al., 2005). Ascorbate is a major primary antioxidant, which reacts chemically with singlet oxygen, superoxide, hydroxyl and thiyl radicals, acting as the natural substrate of many plant peroxidases (Sofo et al., 2005). Glutathione (GSH) is a tripeptide synthesized in the cytosol and the chloroplasts which scavenge singlet oxygen and H_2O_2 and is oxidized to glutathione disulfide (GSSG) when acts as an antioxidant and redox regulator (Sofo et al., 2005).

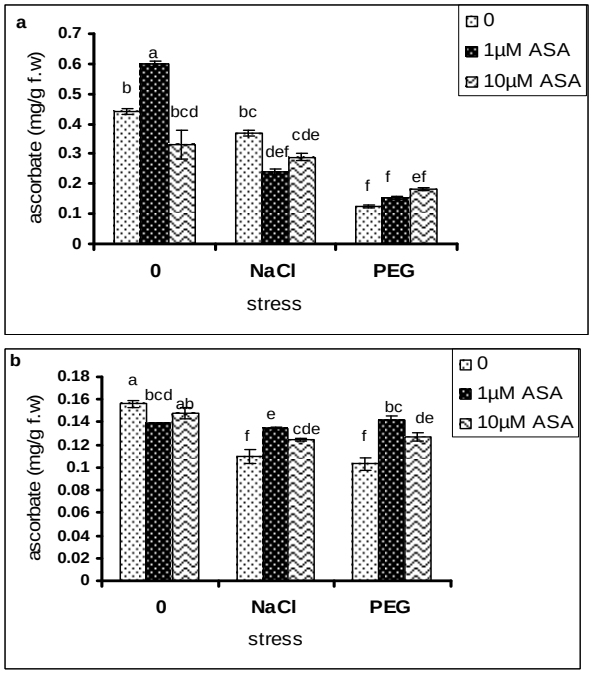

Fig. X. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot ascorbate content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

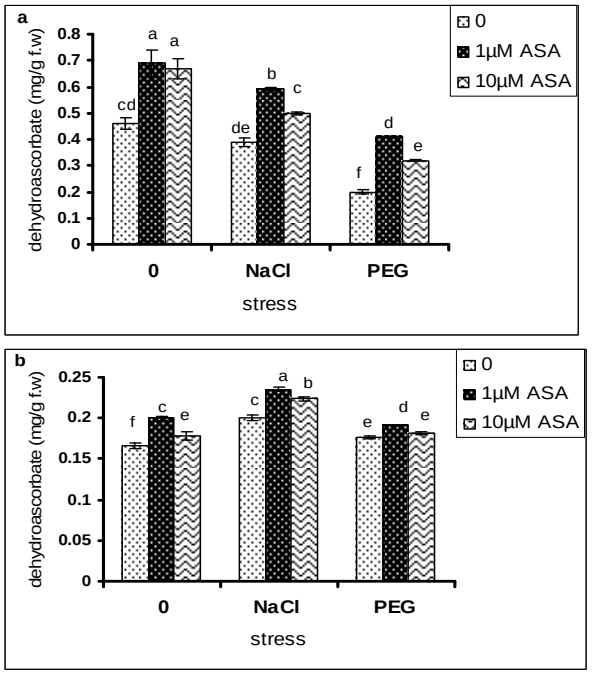

Fig. XI. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot dehydroascorbate content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

In this study, salt and osmotic stress reduced all non-enzymatic compounds in Agria cultivar. Similar results were reported in tomato (Juan et al., 2005), soybean (Abd El Samad and Shaddad, 1997) and Cammelia sinensis (Upadhyaya and Panda, 2004) for carotenoides, in pepper for phenolics (Navarro et al., 2006), in tea for flanovids (Singh et al., 2009), in cotton and spinach for the ascorbates (Parida and Das, 2005; Eraslan et al., 2008) and in naked oat and *Pisum sativum* for glutathione pool (Xu et al., 2008; Iturbe-Ormaexte et al., 1998).

The decrease in carotenoids under stress conditions is due to degradation of ß-carotene and formation of zeaxanthins, which are apparently involved in protection against photoinhibition (Sultana et al., 1999). The reduction of phenolic compounds is normally associated with lower activity of Phenylalanine ammonia-lyase (PAL). In fact, PAL is considered to be the principal enzyme of the phenylpropanoid pathway, catalyzing the transformation by deamination of L-phenylalanine into trans-cinnamic acid, which is the primary intermediary in the biosynthesis of phenolics (Wen et al., 2008; Solecka, 1997; Singh et al., 2009). However, application of PAL inhibitor, 2-amino-2-indanophonic acid (AIfsP), could decrease PAL activity and the concentration of the phenolics was markedly decreased and the resistance to the stresses was weakened (Wen et al., 2008; Solecka, 1997). In this study, the activity of PAL was decreased under salt and osmotic stress and the reduction was higher in PEG than the salt in Agria. Similar to our findings, salt stress reduced PAL activity in citrus plants (Dunn et al., 1998) and drought stress reduced the expression of PAL gene in tea plants (Singh et al., 2009).

Many plants accumulate proline as a non-toxic and protective osmolyte under salt and drought conditions. In present study, proline content was increased under NaCl and PEG stress but the accumulation of proline was greater under PEG induced osmotic stress than that under NaCl induced stress. Similar result was also reported by Aqeel Ahmad et al., (2007) in rice plants. The activities of key enzymes of metabolism including Δ^1 pyrroline-5carboxylate synthetase (P5CS) and proline dehydrogenase (ProDH) were determined under salt and osmotic

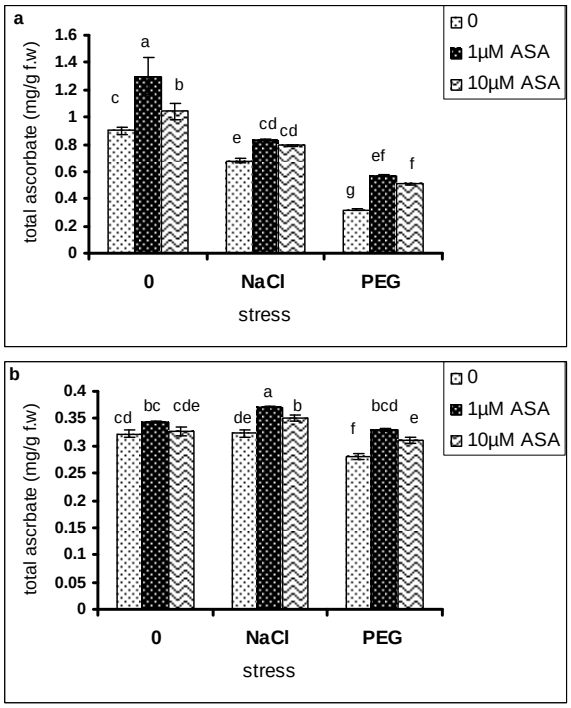

Fig. XII. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot total ascorbate in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

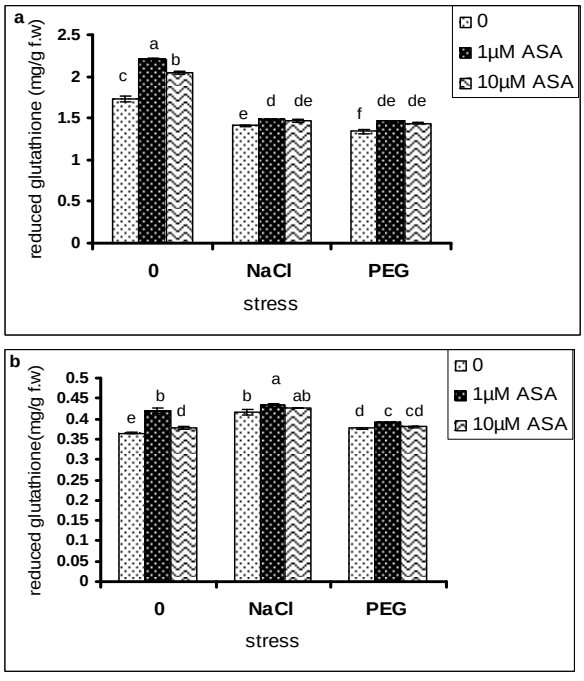

Fig. XIII. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot reduced glutathione content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

stresses. The activity of P5CS, that catalysis the first step in the pathway of conversion of glutamate to proline, was significantly increased in this species under NaCl and PEG stress. The increase in P5CS activity may have contributed to the elevated levels of proline. ProDH converts proline to glutamate. Thus, this enzyme also influences the level of proline. Salt and osmotic stress caused a reduction in the activity of ProDH in this species. Similar to our results, the activity of P5CS increased and the activity of ProDH decreased in some potato cultivars under salt stress (Rahnama and Ebrahimzadeh, 2004) and in cotton genotypes under water stress (Parida et al., 2008).

In our study, response of Agria cultivar and *S. acaule* was completely different to NaCl. Reduction in shoot dry weight in Agria cultivar and no change in *S. acaule* indicated that this species was more tolerant than Agria cultivar. Also considering the lower MDA level and higher PAL activity in *S. acaule* under salt stress, which are two main markers of envinmental stress, would further suggest that there were clear differences in these two species in response to NaCl. Reduction in growth parameters and enhanced level of MDA under salt and osmotic stress has been reported in many crop species (Agarwal and Pandey, 2004; Bandeoglu et al., 2004; Bandurrska and Stroinski, 2005; Demiral et al., 2005; Fazeli et al., 2007; Jain et al., 2006; Korkmaz et al., 2007).

The better growth and performance of *S. acaule* under salt treatment is mainly due to its ability to take up more Na^+ than Agria cultivar (53 vs 22). Therefore, we presumed that this species displayed a halophytic behavior and was able to cope with excess toxic ions by compartmentalizing Na^+ into vacuole. In fact, *S. acaule* exhibited high level in almost all non-enzymatic antioxidants measured under salt treatment, a response which was observed in *Amranthus tricolor* (Parida and Das, 2005), *Kochia prostrata* (Karimi et al., 2005) and *Plantago coronopus* (Koyro, 2006) under salt treatment.

On the contrary, in Agria cultivar, NaCl impaired growth; reduced all non-enzymatic compounds recorded and increased MDA. Although both species were affected by osmotic stress, the relative reduction in shoot dry weight

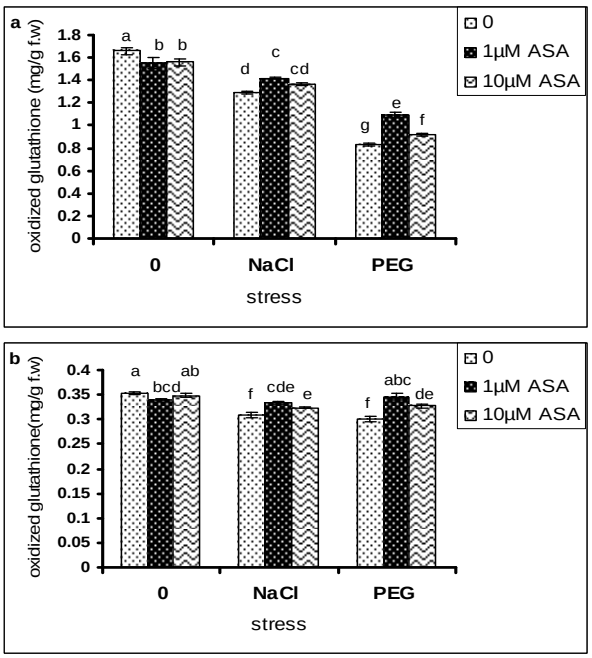

Fig. XIV. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot oxidized glutathione content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

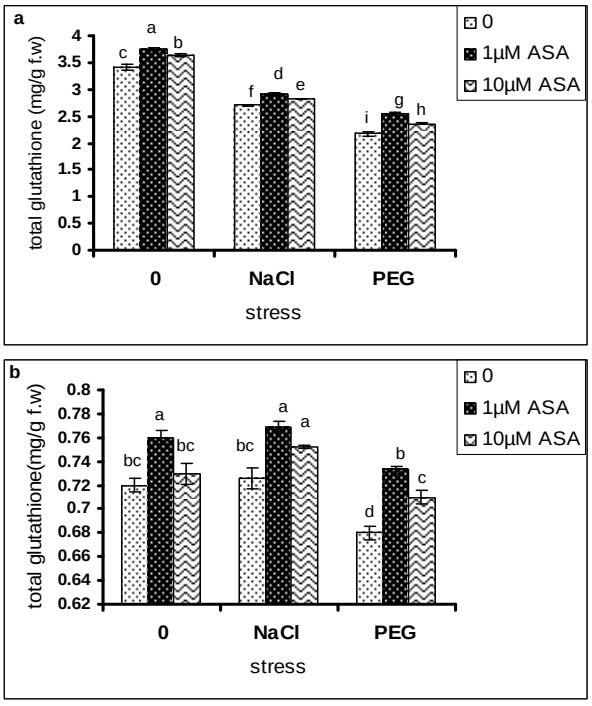

Fig. XV. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot total glutathione in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

and many other antioxidant parameters of wild species was far less than the Agria cultivar. This indicated that this species is also more drought tolerant than the Agria cultivar.

ASA improved plant performance under salt and osmotic stress and to a lesser extend under non- stress conditions in both species. Similar results were reported for barley (El-Tayeb 2005) and maize (Khodary 2004) under salt stress and for wheat under drought stress (Singh and Usha 2003). ASA also reduced the level of lipid peroxidation (MDA) under both types of stress in Agria cultivar and under osmotic stress in *S. acaule* indicating that pretreatment with this chemical facilitated the maintenance of membrane function under stress conditions. Similar pattern of response has been reported in several crop species under abiotic stress conditions (Agarwal et al. 2005; El-Tayeb 2005; Xu et al. 2008).

There was an increase in carotenoids content with application of ASA under all conditions in the current study, indicating an improved potential for growth with ASA treatments. The increase in carotenoids content by SA has been also reported by Eraslan et al. (2007) in carrot, El-Tayeb (2005) in barley and Khodary (2004) in maize. SA also activated the synthesis of carotenoids and xanthophylls in wheat and moong plants (Moharekar et al. 2003). The better growth performances of these species under ASA treatment was associated with an increased level of PAL enzyme activity under stress and non-stress conditions which led to the enhanced levels of non-enzymatic compounds. Most studies have shown that PAL enzyme plays a key role in salicylic acid biosynthesis and exogenous application of salicylic acid could induce the expression of many defense genes including PAL in tobacco, tomato and parsley (Wen et al. 2008) and the expression of PAL gene in grape berry and the accumulation of polyphenols under high temperature (Wen et al. 2008). It was reported that ASA pretreatment increased the level of anthocyanins in carrot (Eraslan et al. 2007) and spinach (Eraslan et al. 2008) under salt conditions and also enhanced the ascorbate and glutathione pool in the stressed and unstressed plants. These compounds may have an important role in

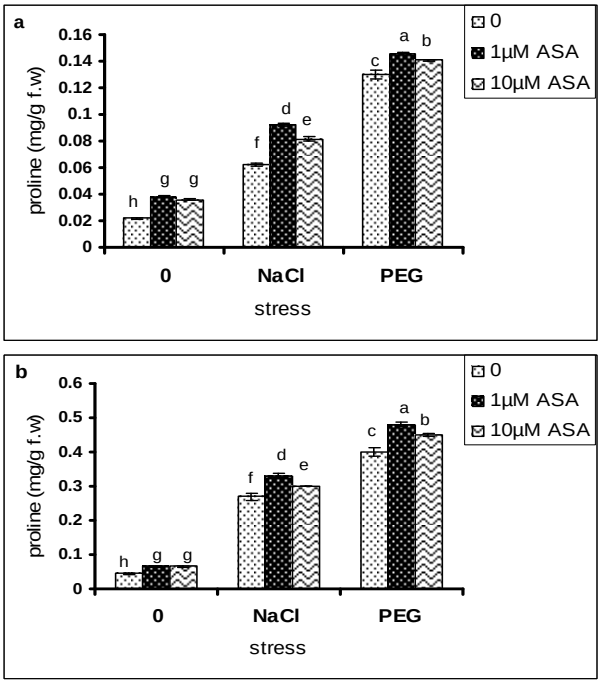

Fig. XVI. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot proline content in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

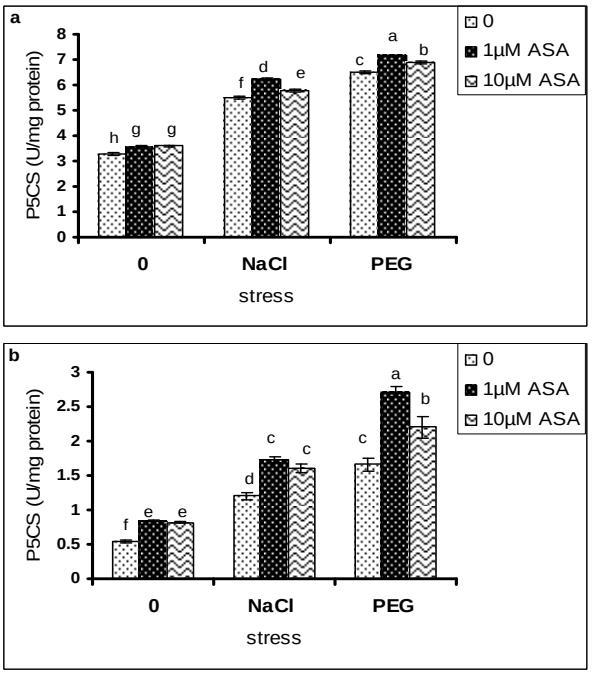

Fig. XVII. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot Δ^1-pyrroline-5-carboxylate synthetase (P5CS) activity in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

protecting cells especially photosynthetic apparatus by scavenging ROS arising during stresses. Xu et al. (2008) have also reported SA-induced increase in ascorbate and glutathione in naked oat under salt stress.

ASA application decreased the severity of NaCl and PEG stresses in both species studied. This agrees with the findings of others that SA induces tolerance to many biotic and abiotic stresses (Horvath et al. 2007a, b; Gue et al. 2007; Senaratna et al. 2000; Sakhabutdinova et al. 2003). When applied at suitable concentration, SA caused transient oxidative stress in plants, which acts as a hardening process, increasing antioxidant capacity of plants (Horvath et al. 2007b). However, to induce antioxidant activity, low concentration of H_2O_2 is required.

As a conclusion, the results of present study revealed that, the higher tolerance of wild species (*S. acaule*) to salt and osmotic stress was due to its ability to cope with the high level of Na^+ in shoot, which may be of special interest concerning the future studies, and has much potential for breeding programs. In addition, the improved performance of treated plants with ASA was associated with enhanced levels of non-enzymatic antioxidants.

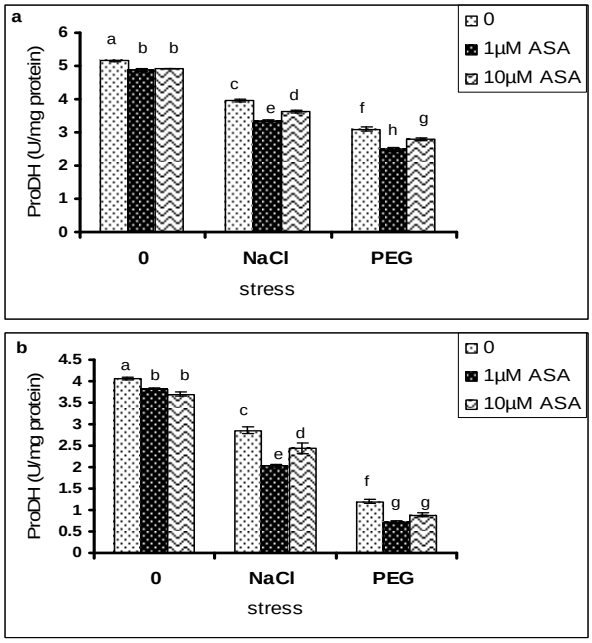

Fig. XVIII. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot Proline dehydrogenase (ProDH) activity in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates.The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

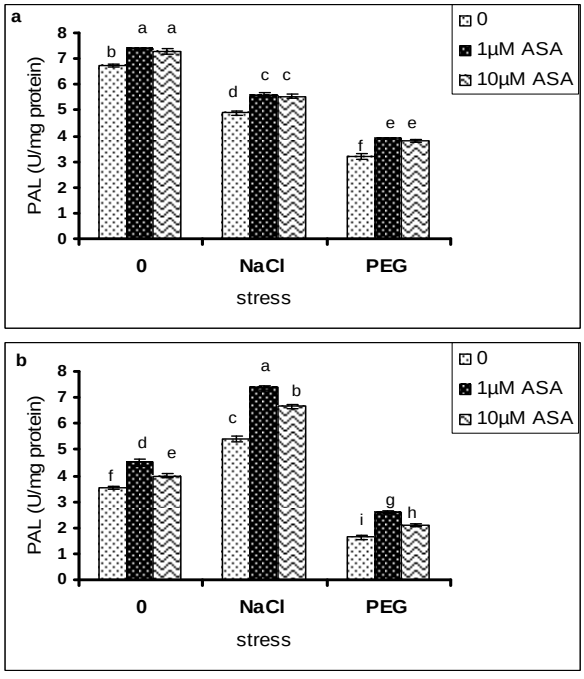

Fig. XIX. The interactive effect of ASA pretreatment and stresses (NaCl and PEG) on the shoot Phenylalanine ammonia-lyase (PAL) activity in *S. tuberosum* cv. Agria (a) and *S. acaule* (b). Results (± SE) are the mean of six replicates. The same letters shows no difference ($p \leq 0.05$) according to Duncan test.

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