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Role of cellular NADP+/NADPH ratio in the acclimative mechanism of two common bean cultivars toward salt stress

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Summary

This study is aimed to evaluate the adaptive mechanism in two common bean (*Phaseolus vulgaris* L.) cultivars, cv. Bronco and cv. Paulista subjected to different concentrations of NaCl (0, 25, 50 and 75 mM). Salt treatments resulted in a significant decrease in the fresh and dry biomasses of shoots and roots as well as leaf relative water content (RWC) in both bean cultivars. Salt stress determines a diversion of plant metabolism towards the synthesis of phenolics, proline and trigonelline (TRG) components together with an increase in the $NADP^+$/NADPH ratio. This increase was accompanied with a significant increase in glucose-6-phosphate dehydrogenase (G-6-PDH) and L-phenylalanine ammonia-lyase (PAL) activities in both bean cultivars. Furthermore, increasing NaCl levels induced oxidative stress measured in terms of malondialdehyde and H_2O_2 contents. Salt stress triggered an increase in guaiacol peroxidae (GPX) activity in both bean cultivars, whereas polyphenol oxidase (PPO) activity increased only in Bronco plants. The results are discussed in the light of possible roles and regulation of cellular redox potential ($NADP^+$/NADPH) in the maintenance of acclimative mechanism in the two common bean cultivars grown under NaCl stress.

Introduction

Salinity is one of the most important abiotic constraints that adversely affect plant growth and development throughout the world. It exerts negative consequences on plant growth, protein biosynthesis and photosynthetic pigments and triggers secondary stresses like oxidative, ionic and osmotic imbalances (MORARI et al., 2015). In addition, salinity increases the production of reactive oxygen species (ROS) leading to oxidative damage and hence cell death (APEL and HIRT, 2004). However, to maintain the level of ROS under tight control, plants develop complex defense mechanisms composed of enzymatic and non enzymatic systems (KAYA et al., 2013). Some enzymatic antioxidants have been reported to increase in response to salinity such as catalase (CAT), guiacol peroxidase (GPX) and polyphenol oxidase (PPO) (KAVAS et al., 2015). The non enzymatic antioxidants include tocopherols, carotenoids and phenolic phytochemicals with the latter serve as phenolic antioxidants due to their ability to donate hydrogen from the hydroxyl groups positioned along their aromatic ring to terminate ROS and other biomolecules (BALASUNDRAM et al., 2006). Several studies showed that increasing water deficit and salt stress resulted in an increase of phenolic compounds in different plant species (ALI and ISMAIL, 2014; ABHILASH-JOSEPH et al., 2015). Synthesis of compatible solutes (e.g. proline, glycine betaine, trigonelline) is one of the most prominent response mechanisms triggered upon salt stress which contribute to stabilize protein structures (CARDI et al., 2015). Trigonelline (TRG; 1-N-methylnicotinic acid) is a member of alkaloids, derived during *de novo* and salvage of pyridine nucleotides in plants (MATSUI et al., 2007). Many legumes produce TRG also as a secondary metabolite such as fenugreek (HASSANZADEH et al., 2011).

It has been reported that glucose-6-phosphate dehydrogenase (G-6-PDH) is a key enzyme for maintenance of redox potential in cells via oxidative pentose phosphate pathway, OPPP (FARHUD and YAZDANPANAH, 2008; SCHARTE et al., 2009). YU et al. (2004) have stated that the oxidative stress and H_2O_2 accumulation induced by the elicitors under environmental stresses could stimulate G-6-PDH activity and enzymes of glutathione cycle. Redox reactions are the fundamental metabolic processes through which cells convert and distribute the energy that is necessary for growth and maintenance. Most stress conditions that impose constraints on plant growth and development involve adjustments in redox state (BUCHANAN and BALMER, 2005). SCHEIBE et al. (2005) reported that in plant stress reactions, NADPH is particularly important as a branch point between ROS-promoting and ROS-processing metabolism. Cytosolic NADPH provides the reductant for production of ROS through NADPH oxidases as well as the protection of cells against ROS by transferring of oxidized glutathione to reduced form *via* glutathione reductase activity (FARHUD and YAZDANPANAH, 2008).

Phenylalanine ammonia-lyase (PAL) is considered the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism. It can be induced by various biotic and abiotic stresses, which result in the accumulation of secondary metabolites such as phenolics, flavonoids, phytoalexins and lignin involved in defense response to protect the plants from stress (KELIJ et al., 2013).

Common bean (*Phaseolus vulgaris* L.) is widely used as protein source with highly nutritive value in human nutrition in many parts of the world including Egypt. It is also a valuable leguminous crop worldwide because of its ability to fixate atmospheric nitrogen into the root nodules in a symbiotic interaction with soil rhizobia.

The present study aimed to investigate the regulatory role of $NADP^+$/NADPH ratio in the response of two cultivars of *Phaseoulus vulagaris* L. (cv. Bronco and cv. Paulista) to salinity. The changes in some growth parameters, proline, TRG and pyridine nucleotides [(NADP(H)], total phenolics, alkaloids and flavonoids as well as GPX , PPO, PAL and G6PDH activities were followed.

Materials and methods

Plant material, growth conditions, and treatments

Common bean *(Phaseoulus vulagaris* L.*)* seeds (cv. Bronco and cv. Paulista) were obtained from the Agricultural Research Center, Giza, Egypt. They were surface sterilized with 2.5 % sodium hypochlorite for 10 min, rinsed with distilled water, and soaked for 24 h at 25 °C in aerated water. To choose suitable concentrations of NaCl, a preliminary experiment was conducted where 20 seeds allocated at random in Petri dishes (15 cm diameter) containing filter paper moistened with 20 ml of 0, 10, 25, 50, 75, and 100 mM NaCl, covered by lid, and incubated at natural environmental conditions (photoperiod of

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16 h/8 h light/dark, 28/23±2 °C light/dark temperature; light intensity PPFD, 23 μmol $m^{-2}s^{-1}$) for 3 days. The germination percentage was calculated as a standard of radicle emergence and the specified concentration of NaCl was determined as 25, 50 and 75 mM NaCl. Seeds of uniform size were sterilized as previously mentioned and they were transferred to plastic pots (15 cm in diameter, 20 cm length with a hole at the bottom) filled with fixed amount of previously acid-washed quartz sand. Twenty seeds were germinated in each pot and the pots were placed under natural environmental conditions (a 16-h photoperiod, 28/23±2 °C light/dark temperature and light intensity of about 23 μmol $m^{-2}s^{-1}$). The water holding capacity was kept at 80% during the whole experimental period. The pots were irrigated using half strength modified Hoagland solution (EPSTEIN, 1972) supplemented with 0, 25, 50 and 75 mM NaCl every two-day interval throughout the whole experiment period. After 21 days, homologous plants were harvested, washed thoroughly from adhering soil particles, gently plotted, dissected to shoots and roots and quickly saved for estimation of the various growth parameters and chemical analyses. All chemical analyses were performed on roots and leaves.

Experimental methods

Growth parameters

The roots and leaves were separated and taken for determination of fresh (FM) and dry biomass (DM). Leaf relative water content (RWC) was determined as described by SILVEIRA et al. (2003) based on the following equation:

$$[(FM - DM) / (TM - DM)] \times 100$$

Where FM is the leaf fresh mass, DM is leaf dry mass (after drying at 80 °C for 48 h) and TM is the turgid mass of leaves (after soaking in water for 4h at room temperature).

Estimation of photosynthetic pigments

The photosynthetic pigments were determined after grinding of leaf samples with acetone following the method described by METZNER et al. (1965).

Estimation of lipid peroxidation, H_2O_2 and proline contents

Hydrogen peroxide content was determined according to the method of VELIKOVA et al. (2000). Lipid peroxidation was monitored by spectrophotometric determination of malondialdehyde (MDA) using thiobarbituric acid (TBA) as described in VALENTOVIČ et al. (2006). The content of MDA was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}. Proline was estimated by the method of BATES et al. (1973). Samples were homogenized in 10 mL 3% (w/v) sulfosalicylic acid, and proline was assayed by the acid ninhydrin method. The absorbance was measured spectrophotometrically at 520 nm and proline was calculated based on μM g^{-1} DM.

Extraction and determination of total phenolic compounds, falvonoids and alkaloids contents

Total phenolic contents of leaves and roots of bean plants were determined using the modified Folin-Ciocalteu spectrophotometric method (MCDONALD et al., 2001). Total flavonoids were extracted and determined by the colorimetric methanolic aluminium chloride method of LUXIMON-RAMMA et al. (2002). The total alkaloids content in the plant samples were extracted and determined by the method described by SINGH et al. (2004).

High-performance liquid chromatographic (HPLC) analysis Trigonelline

For estimation of TRG, samples were homogenized in 80% methanol (ZHENG and ASHIHARA, 2004). The homogenate was centrifuged at 3000 × g for 15 min at 4 °C. The supernatant was filtered through a 30-μm syringe filter, and 50 μL of the filtrate was analyzed using a HPLC system (PerkinElmer series 200 LC and UV/VIS detector 200 LC, USA) equipped with a 5-μm column (Spheri-5 RP-18; 220 × 4.6 mm; Brownlee). The solvent used was a mixture of methanol:water (30:70 v/v) as mobile phase run isocracticaly with a flow rate of 1.0 ml/min. The detector was set at 265 nm for the integration of peak areas after calibration with the external standard and the results were expressed as μg g^{-1} DM.

$NADP^+$ and NADPH

For the analysis of adenine and pyridine nucleotides by HPLC, samples (0.3g) were subjected to either acid extraction using 0.6 M perchloric acid (for measurement of ATP, ADP, nicotinamide adenine dinucleotide phosphate ($NADP^+$) and nicotinamide adenine dinucleotide (NAD^+) or alkaline extraction using 0.5 M potassium hydroxide (for measurement of NADPH and NADH). The extract was centrifuged at 10,000 × g at 4 °C for 10 min followed by neutralization with either 0.5 M KOH or 1 M KH_2PO_4 and re-centrifugation at 10,000 × g at 4 °C for 10 min to remove the precipitate. Twenty μl of the resulting supernatants were used for HPLC analysis, as described by CARUSO et al. (2004) using the previously mentioned HPLC system. The mobile phase consisted of potassium phosphate 100 mM (solvent A) at pH 6.0 and methanol (solvent B) (10% A - 30% B) up to 15 min and (30% A - 10% B) for 5 min at flow rate of 0.8 ml/min, UV/VIS detector set at 260 nm for the integration of peak areas after calibration with the external standard and the results were expressed as μg g^{-1} DM.

Enzymes assay

L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5)

L-Phenylalanine ammonia-lyase was extracted according to the method of MORELLO et al. (2005). Samples (300 mg) were homogenized in chilled 0.05 M potassium phosphate buffer (pH 6.6) containing 0.2 g of Triton X-100. The homogenate was centrifuged at 5,000 × g for 15 min and the supernatant was used for enzyme activity assay. The PAL activity was assayed according to the method of CHEN et al. (2006). The cinnamic acid yield was estimated by measuring the absorbance at 290 nm using spectrophotometer (Jenway, 6305, UK). One unit of enzyme activity equals the amount of PAL that produced 1 μmol of cinnamic acid per hour. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient (9630 M^{-1}) (KAGALE et al., 2004) and the results were expressed as μmol cinnamic acid $h^{-1}g^{-1}$ DM.

Glucose-6-phosphate dehydrogenase activity (G-6-PDH, EC 1.1.1.49)

Activity of G-6-PDH was assayed according to the method of ASHIHARA and KOMAMINE (1976). Fresh plant material (0.5 g) was homogenized in 0.05 M Tris-HCl buffer pH 7.0. The homogenate was centrifuged at 5,000 × g for 20 min at 4 °C. G-6-PDH activity was calculated using the extinction coefficient of the NADPH (6.22 mM^{-1} cm^{-1}) and was expressed in mmol NADPH min^{-1} g^{-1} DM.

Antioxidant enzyme assays

For estimation of antioxidant enzymes activity, frozen plant tissues were homogenized in ice-cold 0.1 M potassium phosphate buffer (pH 6.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 15,000 *g* for 20 min at 4 °C. After centrifugation, the supernatant was used for determination of guaiacol peroxidase (GPX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.1) activities. GPX was assayed according to the method of URBANEK et al. (1991) where guaiacol oxidation to tetraguaiacol was followed for 5 min at

470 nm. GPX activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} and was expressed as μmol guaiacol g^{-1} min^{-1}. Polyphenol oxidase (PPO, EC 1.10.3.1) was assayed following the method described by KUMAR and KHAN (1982), where the formation of the purpurogallin was followed at 495 nm. PPO activity was expressed in U min^{-1} g^{-1} DM.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as means ± standard error (SE). The data of all experiments were analyzed using SPSS-16 statistical software. Mean difference comparison of treatments was done by ANOVA and the least significant differences (LSD) at level of $P \leq 0.05$.

Results

Growth parameters

Increasing the salinization of nutrient solution with NaCl resulted in a significant reduction of FM and DM of shoots and roots of both tested common bean cultivars, which was in parallel with a significant decrease in RWC of leaves (Tab. 1). The reduction was more obvious in Paulista than in Bronco, for example the RWC of leaves of 50 mM NaCl-stressed Bronco and Paulista plants were 70 % and 47 % respectively, compared to controls.

Photosynthetic pigments

The total pigment content significantly decreased with increasing NaCl level. This decrease was mainly related to Chl *a* especially at high NaCl level. On the other hand, it was clear that the trend of carotenoids content was greatly different according to the cultivar type (Tab. 2). In cv. Bronco, carotenoids content was significantly increased with increasing salinity levels, while in cv. Paulista they significantly decreased at moderate and highest salinity levels. Although, variation trend of carotenoids content was noted, the Carot./Chl.a+b increased significantly by increasing salinity levels in the nutrient medium, particularly in cultivar Paulista

Lipid peroxidation, H_2O_2 and proline contents

Increasing NaCl concentrations in the nutrient medium of bean plants resulted in a significant accumulation of H_2O_2 in the leaves and roots of bean cultivars in response to salinity levels (Fig. 1). At highly-stressed Bronco plants, the H_2O_2 concentration in the leaves and roots was 2.6-and 3.0-fold of untreated plants, respectively. The corresponding values in Paulista plants were 4.0-and 5.1-fold respectively. Parallel to changes in H_2O_2, increasing NaCl concentrations in the nutrient medium resulted in a significant increase of endogenous MDA in the leaves and roots of bean plants. In 75 mM NaCl-treated 21-d old Bronco plants, MDA content in the leaves and roots was 3.3-and 6.6-fold of control respectively. The corresponding values in Paulista plants were 5.6- and 11.3-fold respectively (Fig. 1).

Data in Fig. 2 clearly demonstrated that proline content significantly increased with increasing NaCl concentrations in the rooting nutrient medium; this increase was markedly greater in the leaves and roots of cv. Bronco than in the Paulista one.

Total phenolic, falvonoids and alkaloids contents

There was a significant increase of phenolic compounds in the leaves and roots of both cultivars in response to salt stress; the accumulation in the cv. Paulista was greater than in the cv. Bronco (Tab. 3). At high NaCl level in nutrient medium, the total phenolics content of leaves and roots of Bronco plants was 2.7 and 4.2-fold of unstressed plants, respectively. In Paulista plants these values were 5.5 and 9.9-fold the control, respectively. The same trend was observed for total flavonoid

Tab. 1: Changes in fresh and dry biomasses of roots and shoots as well as leaf relative water content (RWC) of bean plants (cv. Bronco and cv. Paulista) in response to salinity in response to salinity. Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Bean cultivar	Treatment	FM		DM		RWC
		roots	shoot	roots	shoot	leaves
cv. Bronco	Control	381^{a}±4.4	551^{a}±3.1	86^{a}±1.5	119^{a}±2.2	80.1^{a}±1.4
	25 mM NaCl	301^{a}±3.5	466^{ab}±3.6	77^{a}±1.7	93^{a}±1.1	73.9^{b}±0.76
	50 mM NaCl	138^{b}±3.8	242^{b}±4.1	62^{b}±1.5	66^{b}±1.6	56.0^{c}±0.98
	75 mM NaCl	71^{b}±1.0	118^{c}±2.1	35^{c}±0.87	49^{b}±1.7	41.3^{d}±0.72
cv. Paulista	Control	321^{a}±3.2	439^{a}±6.0	82^{a}v±1.4	99^{a}±2.1	85.3^{a}±1.16
	25 mM NaCl	141^{b}±3.2	209^{b}±4.4	60^{b}±0.87	76^{a}±1.4	69.1^{b}±0.85
	50 mM NaCl	68^{c}±1.1	94^{c}±1.6	41^{b}±0.93	46^{b}±0.87	39.8^{c}±0.5
	75 mM NaCl	28^{d}±1.1	56^{d}±1.1	19^{c}±0.55	29^{b}±0.79	25.2^{d}±0.52

Tab. 2: Change in photosynthetic pigments (mg g^{-1} DM) in the leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity. Chl.: chlorophyll; carot.: carotenoids**.** Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Treatment	cv. Bronco				cv. Paulista			
	Chl.a	Chl.b	Carot.	Cart./(a+b)	Chl.a	Chl.b	Carot.	Cart./(a+b)
Control	98.03^{a}±1.8	40.17^{a}±0.68	19.59^{b}±0.33	0.144^{d}	94.03^{a}±1.1	42.35^{a}±0.44	24.18^{a}±0.39	0.177^{c}
25 mM NaCl	72.75^{b}±1.5	39.58^{a}±1.03	21.11^{a}±0.45	0.188^{c}	88.89^{b}±1.49	32.74^{b}±0.62	23.30^{a}±0.36	0.196^{c}
50 mM NaCl	59.01^{c}±1.26	26.14^{b}±0.38	24.68^{c}±0.65	0.290^{b}	38.01^{c}±0.9	19.36^{c}±0.40	19.25^{b}±0.26	0.336^{b}
75 mM NaCl	46.60^{d}±1.02	11.99^{c}±0.44	24.69^{c}±0.38	0.421^{a}	20.24^{d}±0.33	10.56^{d}±0.23	14.98^{C}±0.32	0.486^{a}

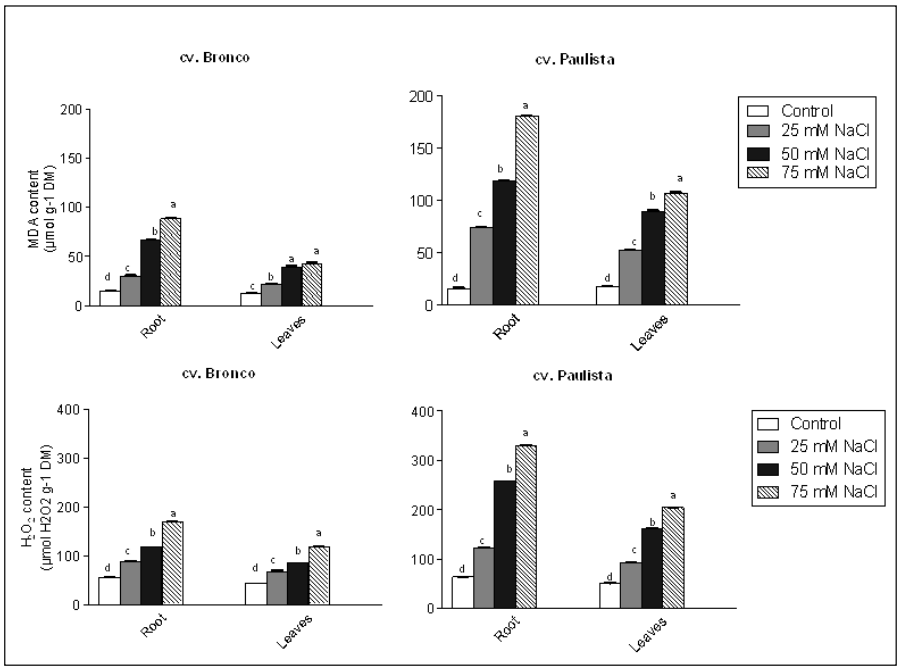

Fig. 1: Changes in hydrogen peroxide (H_2O_2) content (µmol H_2O_2 g^{-1} DM) and malondialdehyde (MDA) content (µmol g^{-1} DM) in the roots and leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity. Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

content. As far as alkaloid content is concerned, increasing NaCl in the nutrient solution resulted in a significant increase of alkaloids content in leaves and roots of cv. Bronco only, compared to corresponding control (Tab. 3). Conversely, moderate and higher NaCl levels led to significant decrease of alkaloids in leaves and roots of Paulista plants compared to corresponding control.

Trigonelline, NADP+/ NADPH ratio

In cv. Bronco, there was a significant increase in TRG content in the leaves and roots in response to increasing NaCl concentration. The opposite trend was observed in cv. Paulista. At 75 mM NaCl, the increase in TRG content in the leaves and roots of Bronco plants amounted 126% and 59%, respectively compared to control. In cultivar Paulista, TRG content decreased by 31% and 27% in the leaves and roots of highly NaCl-stressed plants, respectively (Fig. 2).

The $NADP^+$ and NADPH content (Tab. 4) in the leaves and roots of Bronco and Paulista plants showed variable changes in response to cultivar and NaCl concentrations. However, the ratio of $NADP^+$/ NADPH in the leaves and roots of both bean cultivars was markedly increased with increasing NaCl levels; the increase in Paulista was lower than that in the Bronco plants. The $NADP^+$/NADPH ratio in the leaves and roots of unstressed Bronco plants increased from 0.347 and 0.626 to 1.546 and 1.745 respectively, in 75 mM NaCl-stressed plants. In Paulista plants these values increase from 0.334 and 0.640 to 0.938 and 1.506 respectively.

Enzyme activities

The activity of PAL enzyme significantly increased in response to increasing NaCl level (Tab. 5). The highest increase was recorded in the leaves and roots of 75 mM NaCl-stressed Bronco plants and recorded 4.9- and 2.8-fold of corresponding control. The corresponding values in Paulista plants were 2.7-and 2.2-fold respectively.

Glucose-6-phosphate dehydrogenase activity increased significantly in the leaves and roots of cv. Bronco and cv. Paulista with increas-

Tab. 3: Changes in total phenolic (mg gallic acid eq·g^{-1} DM), flavonoids (µg g^{-1} DM) and alkaloids (µg g^{-1} DM) contents in the roots and leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity. Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Bean cultivar	Treatment	Total phenolics		Total flavonoids		Total alkaloids	
		roots	leaves	roots	leaves	roots	leaves
cv. Bronco	**Control**	557^{d}±8.7	441^{d}±3.8	69^{d}±1.80	574^{d}±8.1	55^{d}±0.81	106^{d}±0.96
	25 mM NaCl	1228^{c}±7.2	821^{c}±10.6	85^{c}±1.50	643^{c}±6.8	64^{c}±0.84	139^{c}±1.42
	50 mM NaCl	1870^{b}±18.0	1087^{b}±16.8	97^{a}±1.80	893^{a}±6.8	95^{b}±1.35	202^{b}±1.71
	75 mM NaCl	2356^{a}±34.2	1175^{a}±23.6	102^{a}±1.90	812^{b}±8.2	108^{a}±1.36	231^{a}±2.36
cv. Paulista	**Control**	371^{d}±5.3	393^{d}±4.6	53^{c}±1.30	327^{d}±3.2	49^{a}±0.66	97^{a}±1.16
	25 mM NaCl	1484^{c}±21.60	1294^{c}±20.8	78^{ab}±2.10	533^{c}±3.6	51^{a}±0.78	99^{a}±1.21
	50 mM NaCl	2186^{b}±22.0	1510^{b}±18.0	80^{ab}±2.70	641^{a}±4.7	37^{b}±0.46	81^{b}±1.15
	75 mM NaCl	3676^{a}±43.4	2142^{a}±51.4	86^{a}±1.80	580^{c}±4.1	33^{b}±0.71	73^{b}±1.07

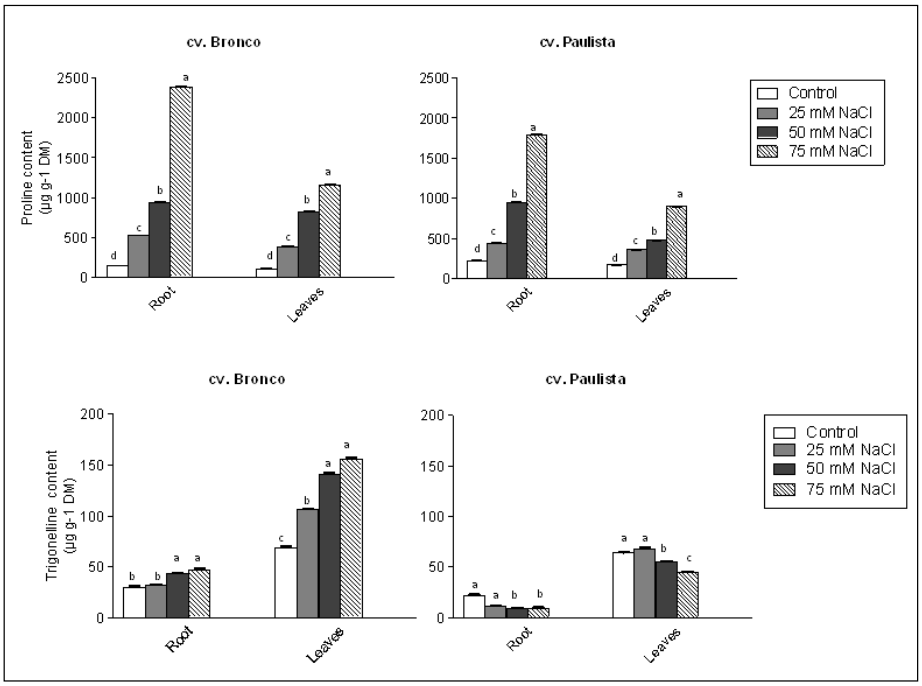

Fig. 2: Changes in proline (µg g^{-1} DM) and trigonelline content (µg g^{-1} DM) in the roots and leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity. Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Tab. 4: Changes in $NADP^+$, NADPH contents (µM) and $NADP^+$/ NADPH ratio in the roots and leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity.

Treatment	cv. Bronco					
	Roots			Leaves		
	$NADP^+$	NADPH	$NADP^+$ / NADPH	$NADP^+$	NADPH	$NADP^+$ / NADPH
Control	5.853	9.343	0.626	13.633	39.210	0.348
25 mM NaCl	35.246	33.646	1.048	15.432	22.617	0.682
75 mM NaCl	5.414	3.102	1.745	5.448	3.523	1.546
Treatment	**cv. Paulista**					
	$NADP^+$	NADPH	$NADP^+$ / NADPH	$NADP^+$	NADPH	$NADP^+$ / NADPH
Control	36.442	56.968	0.640	9.080	27.174	0.334
25 mM NaCl	5.432	5.867	0.926	3.678	9.749	0.377
75 mM NaCl	5.767	3.618	1.594	12.922	13.780	0.938

ing salinity levels of the nutrient medium (Tab. 5); the activity in the former was greatly lower than in the latter. The highest increase was recorded in the leaves of 75 mM NaCl-stressed Paulista plants; 4.5-fold in compare to control.

Increasing the salinization of nutrient solution with NaCl resulted in a significant increase in GPX activity in the two tested bean cultivars (Fig. 3). It is noteworthy that GPX activity in the leaves and roots of NaCl-stressed Paulista plants was greater than in Bronco plants. The GPX in the leaves of 50mM NaCl-stressed Paulista plants was 2.9-fold of unstressed plants. The corresponding value for Bronco plants was 1.6-fold.

PPO activity in the leaves and roots of Bronco plants was significantly increased in response to NaCl treatment (Fig. 3). In contrast, the PPO activity in the leaves and roots of Paulista plants decreased significantly at high salinity level. The decrease of PPO activity in 75 mM NaCl-stressed Paulista leaves and roots was 67% and 73% respectively, compared to control.

Discussion

The drastic effect of NaCl on the growth and leaf RWC of both bean cultivars, particularly Paulista plants might be attributed to increasing ROS generation and hence disturbance of plasma membrane integrity and water uptake as well as osmotic imbalance. Srivastava et al. (2015) have reported that salt stress stimulate the formation of ROS leading to oxidative stress. In agreement with this view, there was a significant increase in H_2O_2 and MDA contents in salt stressed Paulista plants more than in Bronco one revealing the sensitivity of Paulista plants to salt stress. Similar observations were recorded for several plant species such as *Arachis hypogaea* (Kavas et al., 2015). In the present study, the content of photosynthetic pigment significantly declined in both bean cultivars in response to salt stress except carotenoid content, which was increased only in Bronco plants. These observations were accompanied by a significant increase in H_2O_2 and MDA contents suggesting an enhancement of oxidative stress causing a destructive effect on the photosynthetic machinery.

Tab. 5: Changes in glucose -6-phosphate dehydrogenase (G-6-PDH) (μmol g^{-1} DM min^{-1}) and phenylalanine ammonia-lyase (PAL) activities (μmol cinnamic·g^{-1} DM min^{-1}) in the roots and leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity. Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

Bean cultivar	Treatment	G-6-PDH		PAL	
		roots	leaves	roots	leaves
cv. Bronco	Control	1.76^{c}±0.03	1.87^{b}±0.03	35.92^{c}±1.00	33.29^{d}±2.01
	25 mM NaCl	1.84^{bc}±0.04	2.59^{b}±0.10	42.46^{c}±0.50	90.78^{c}±1.04
	50 mM NaCl	2.65^{ab}±0.05	4.78^{a}±0.08	75.40^{b}±0.40	109.12^{b}±1.00
	75 mM NaCl	3.48^{a}±0.07	4.82^{a}±0.10	98.98^{a}±1.00	162.15^{a}±2.04
cv. Paulista	Control	1.69^{d}±0.10	1.77^{b}±0.11	31.98^{d}±1.02	38.85^{c}±2.01
	25 mM NaCl	5.30^{c}±0.10	3.98^{b}±0.20	40.32^{c}±2.02	86.78^{b}±1.01
	50 mM NaCl	5.88^{bc}±0.12	6.45^{a}±0.50	58.92^{b}±2.02	90.30^{b}±1.90
	75 mM NaCl	6.54^{ab}±0.31	7.98^{a}±0.15	69.96^{a}±1.03	105.58^{a}±2.02

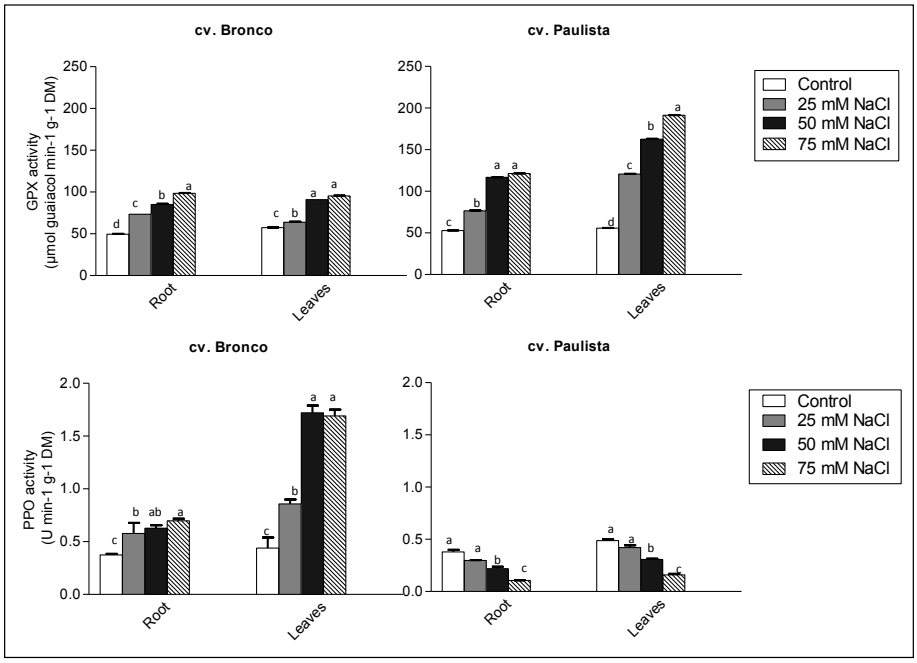

Fig. 3: Changes in guaiacol peroxidase (GPX) activity (μmol guaiacol min^{-1} g^{-1} DM) and polyphenol oxidase (PPO) activity (U min^{-1} g^{-1} DM) in the roots and leaves of bean plants (cv. Bronco and cv. Paulista) in response to salinity. Values are the means of 3 independent replicates ± SE; means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD).

The suppression in photosynthetic pigments content has been reported to be related to the inhibition of specific enzymes responsible for their synthesis and induction of some degradative enzymes such as chlorophyllase (Fang et al., 1998) as well as destruction of the photosynthetic machinery (Yamane et al., 2008).

In response to increasing oxidative damage by generated ROS due to increasing salinity levels in the nutrient medium, both bean cultivars have increased the car/chl a+b ratio, particularly in cultivar Paulista, denoting a development of an adaptive mechanism for removing ROS and reducing the possibility of further damage of photosynthetic apparatus. It was obvious that carotenoids decreased in cultivar Paulista in response to salinity level. But when it is compared as a ratio from total chlorophyll it was found increased, which denotes an initial development of adaptive mechanism in this cultivar. De Pascale et al. (2001) reported that carotenoids react with lipid peroxidation product to terminate chain reactions, while Loggini et al. (1999) suggested that carotenoids directly react with singlet oxygen.

Free proline is primarily localized in the cytosol and plastids (Kavi Kishor and Sreenivasulu, 2014). Both proline and TRG are low molecular-weight quaternary QAC accumulated in cells of higher plants in response to osmotic stress and acting as osmoprotectant and ROS scavengers (Cardi et al., 2015). There was a significant accumulation of proline and TRG in the leaves and roots of cv. Bronco in response to salinity while in cv. Paulista, increasing NaCl levels lead to a significant decrease in TRG content in both roots and leaves. These results might reveal the involvement of proline and TRG (as osmolyte) in mediating osmotic adjustment by preventing the dehydration of protoplasm, protecting sub-cellular structures and membranes as OH^{-} scavenger and stabilizing protein (Demiral and Turkan, 2006). Several authors have reported the accumulation of proline and TRG in several plant species grown under salinity conditions (Dawood et al., 2014) while other authors did not observe any appreciable increase in free proline content in response to salinity stress (Taffouo et al., 2009). Rivero et al. (2004) reported that the accumulation of proline in salt-stressed plant organs might be due to enhancement of NADPH-pyrroline-5- carboxylate synthetase. In

the present study, the accumulation of proline and TRG in Bronco plants was accompanied by a higher RWC and lower H_2O_2 and MDA contents compared to cv. Paulista, which might reveal the role of proline and TRG as osmoregulator and antioxidant compound i.e. cv. Bronco might be a salt-adaptive cultivar. In this respect HARE et al. (1998) noted that proline and TRG biosyntheses maintain appropriate NAD^+/NADH and $NADP^+$/NADPH ratios compatible with metabolism. Data recorded in this study demonstrated that $NADP^+$/NADPH ratio in the leaves and roots of NaCl-stressed Bronco was higher than those in Paulista plants revealing the consumption of NADPH as H-donner in proline and TRG biosynthesis. This might reflect the role of cytosolic $NADP^+$/NADPH in the development of an adaptive mechanism via biosynthesis of osmoregulators. NADPH is a key cofactor in cellular redox homoeostasis. Thus, the switch of $NADP^+$/NADPH might make a sense in particular situation during subjection of both Bronco and Paulista plants to salt stress, when metabolism shifts to synthesizing defense compounds, such as phenolics, proline and TRG. Moreover, the enhancement of adaptive mechanisms against generated ROS in salt-tolerant Bronco plants might indicate that NADPH acts as a cofactor for other antioxidant enzymes, such as glutathione reductase (FARHUD and YAZDANPANAH, 2008). Furthermore, nicotinic acid (NA) that is produced during pyridine nucleotide cycle in plants is known as a precursor of TRG biosynthesis (MATSUI et al., 2007). KHANAM (2007) reported that when TRG accumulated, it is considered as a detoxifying agent for excess NA and nicotinamide released from NAD cycle. SCHARTE et al. (2009) concluded that the increase in NADPH might induce the activity of NADPH-oxidase enzyme and hence increasing ROS generation. Thus, in addition to its role as an osmoregulator the increase in TRG content in salt-adaptive Bronco plants could be related to detoxification as well as shifting off suppression of growth caused by excess NA during NAD cycle, whereas in salt-sensitive Paulista plants the decrease in TRG content might be attributed to increased NADPH-oxidase activity and increase in H_2O_2 content (Fig. 1).

Osmoregulation is not the sole response to salinity, as this stress is also counteracted by generation of secondary metabolites and enhancing several antioxidant enzymes. Total phenolics and flavonoids significantly increased in both bean cultivars in response to salinity level (Tab. 3). These results were associated with an enhancement of GPX activity revealing the adaptive role of these secondary metabolites against salt stress. Furthermore, the activity of PPO in Bronco plants was significantly increased in response to salinity while it decreased in plants of cv. Paulista. These observations might be explained by a marked reduction of generated ROS in NaCl-stressed Bronco plants using phenolic compounds as H-donors to shift off the inhibitory effect of generated H_2O_2 and the toxic effect of accumulated phenolics and hence improve the growth more than in Paulista plants; in other words cv. Bronco may be a salt-tolerant or salt-acclimative bean cultivar. The increase in total phenolics and flavonoid contents is in conformity with the findings in *Vicia faba* (DAWOOD and EL-AWAD, 2015). In addition, increased GPX and PPO activities in response to salinity has been reported by many authors (BIAN and JIANG, 2009; DAWOOD et al., 2014).

It has been reported that upon exposure to salt, the cytosolic G6-PDH activity may increase in order to support the syntheses of cofactors (as reducing power, NADPH) or intermediates (as phenolics) involved in the tolerance mechanisms (DAL SANTO et al., 2012). The cytosolic G6-PDH activity significantly increased in the leaves and roots of both bean cultivars in response to salinity. These results denote the enhancement of OPPP to generate the precursors of phenolic compounds via shikimate and phenylpropanoid pathways. Moreover, it was clearly demonstrated that PAL activity in the leaves and roots of both bean cultivars subjected to different concentrations of NaCl increased significantly compared to the control. In accordance with these results, many authors have shown a positive correlation between PAL and total phenolics content in various plants grown under salt stress, such as *Salvia* sp. (VALIFARD et al., 2015). Furthermore, the enhancement of G6-PDH activity in the salt-treated bean plants might reflect the increase in NADP/NADPH ratio under salt stress due to the involvement of produced NA cofactor (NADPH) in the biosynthesis of proline and TRG.

Conclusions

The salt-tolerance ability of two common bean cultivars namely Bronco and Paulista has been compared. The findings reveal better regulation of cytosolic NADP/NADPH ratio in Bronco plants, in which the enhancement of G6-PDH activity under salt stress resulted in the generation of antioxidant components, such as phenolics, falvonoids and TRG as well as osmoregulatory components (proline and TRG) and hence protect the plasma membrane integrity and keep water conservation. Thus, the varied response of cv. Bronco and cv. Paulista to studied NaCl levels might be related to their ability to maintain a cellular redox homoeostasis.

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