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Influence of salinity and adaptive compounds on oxidative stress and antioxidant system in broad bean cultivars contrasting in salt tolerance

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Abstract

Hydrogen peroxide, lipid peroxidation and electrolyte leakage were increased with an increase in salinity; levels for these parameters being lower in the tolerant cultivar (Giza 843) than those in the sensitive one (Giza 716). Treatment with salicylic acid, proline, ascorbic acid, reduced glutathione, in general, variably reduced the levels of these parameters, while combination of these compounds with NaCl partially counteracted the accumulation of H_2O_2 and the increased lipid peroxidation and electrolyte leakage; SA appeared the most inductive. In contrast with a decrease in anthocyanins, total phenols, ascorbic acid and reduced glutathione contents were increased in response to salinization; the magnitude of response being higher with high NaCl. Application of adaptive compounds, either alone or in combination with NaCl, caused a significant increase in the contents of total phenols, AsA and GSH as well as variable changes in anthocyanin contents in relation to controls. The increased values appeared most pronounced with SA and with 300 mM NaCl. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX) and polyphenol oxidase (PPO) increased significantly in both cultivars treated with 50 mM NaCl whereas at 300 mM NaCl, a significant decrease was observed in the activities of SOD, CAT, POX and PPO below the control levels; while the activities of APX and GR increased. Treatment with each of the adaptive compounds either alone or in combination with each salinity level, significantly increased the activities of all the antioxidant enzymes in both the cultivars of broad bean seedlings; SA appeared to be the most effective. The results of the present study indicate the beneficial effect of the used adaptive compounds on salinity tolerance and the best response was observed with salicylic acid.

Keywords: *Vicia faba*, salinity, adaptive compounds, hydrogen peroxide, lipid peroxidation, electrolyte leakage, antioxidants.
Abbreviations: Pro_proline; GSH_glutathione reduced; AsA_ascorbic acid; SA_salicylic acid; SOD_superoxide dismutase; CAT_ catalase; APX_ascorbate peroxidase; GR_glutathione reductase; POX_peroxidase; PPO_polyphenol oxidase; MDA_ malondialdehyde.

Introduction

Salinity is a widespread environmental stress that affects almost every aspect of the physiology and biochemistry of plants and significantly reduces the yield. A metabolic response to salt stress is the synthesis of compatible osmolytes. These mediate osmotic adjustment and therefore protect sub-cellular structures and reduce oxidative damage caused by free radicals (Apel and Hirt, 2004; Parida and Das, 2005; Younis et al., 2009 and 2010).

Oxidative stress is initiated by reactive oxygen species (ROS) which are produced and accumulated during normal aerobic metabolism. Failure to quench or inactivate the ROS may lead to the degradation of macromolecules in the cells as membrane lipids, proteins and nucleic acids (Mittler, 2002; Lee et al., 2004). Of the main symptomatic parameters for oxidative stress developed in response to high salinity, we may refer to H_2O_2 production. Due to the imbalance in ROS production and antioxidant activity, H_2O_2 level increases with increasing salinity in different plants (Asada, 1994). A rapid decline in H_2O_2 concentration in various plant tissues has been observed in response to exogenous application of various adaptive compounds (Gunes et al., 2007; Hossain and Fujta 2010; Azzedine et al., 2011).

Furthermore, of the expected consequences of stress-induced cellular build-up of ROS is an increase in lipid peroxidation inducing a peroxidative damage to plasma membranes associated with electrolyte leakage, in response to high salinity (Hanaa et al., 2003; Koca et al., 2007). Counteraction of these concurrent effects was operative due to treatment with adaptive compounds (Shalata and Neumann, 2001; Popova et al., 2003; Gunes et al., 2007; Khattab, 2007).

Plants possess a battery of antioxidative mechanisms to scavenge the ROS. These include non-enzymatic antioxidants like AsA, GSH ,α-tocopherol and carotenoids and enzymatic antioxidants such as SOD, APX, CAT and GR (Apel and Hirt, 2004; Parida and Das, 2005; Younis et al., 2010). Counteraction of the adverse effects of salinity can be managed by the exogenous application of compatible solutes, antioxidants and plant growth regulators (e.g. Shalata and Neumann, 2001; Gunes et al., 2007; Khattab, 2007; Younis et al., 2009 and 2010). Recent studies have also highlighted the potential role of flavonoids, phenylpropanoids and phenolic acids as effective antioxidants (Michalak, 2006).

Increasing tolerance to salinity stress in crop plants appears necessary in order to increase productivity with limited water supplies and high salinity in Egypt. In the first part of our investigation (Younis and Tourky, 2014), it was observed that treatment of salt-sensitive and salt-tolerant bean seedlings with low or high salinity levels induced significant increases in glucose, proline and glycine with concurrent decreases in sucrose, polysaccharides and nucleic acids, after 14 days of germination, the magnitude of response was more

pronounced under high salinity level. On the other hand, the use of the adaptive compounds, either alone or in combination with each of the salinity levels, induced varied increases in glucose, Pro, glycine, DNA and RNA and decreases in sucrose and polysaccharide contents.

The aim of this study was thus to investigate the possible effects of low and high levels of salinity, either alone or in combination with each of the 4 selected adaptive compounds, on the efficiency of the antioxidant system in germinating susceptible and resistant varieties of *Vicia faba*; a crop plant of great economic importance in Egypt as well as in many other developing and industrialized countries.

Results and Discussion

Changes in hydrogen peroxide

As compared with controls, the hydrogen peroxide content appeared to increase with an increase in the salinity level, both in sensitive and tolerant broad bean cultivars after 14 days (Table 1); the determined contents in the tolerant being lower than that in the sensitive cultivar. Exogenous application of each adaptive compound to both bean cultivars, appeared to induce either a decrease (with AsA and SA) or an increase (with Pro and GSH) in H_2O_2 content below and above the control content, respectively.

Combination of the adaptive compounds with each of the used levels of NaCl induced a marked increase in the hydrogen peroxide content of both sensitive and tolerant bean seedlings above those levels maintained in response to the sole treatment with the respective adaptive compound. The obtained levels were higher in the salt-sensitive than in the salt-tolerant broad bean seedlings. Furthermore, counteraction of the adverse effects induced by each level of NaCl appeared operative in response to treatment with each adaptive compound. The magnitude of counteraction, appeared higher in the salt-tolerant than that obtained in the salt-sensitive bean seedlings (Table 1).

Molecular oxygen in its ground state does not directly cause damage to living cells but when it receives extra energy or electrons, it generates a variety of ROS which will cause oxidative damage to various components of living cells including lipids, proteins and nucleic acids (Mittler, 2002; Apel and Hirt, 2004 ; Parida and Das, 2005). The most common ROS generated under normal conditions are $^{\cdot}O_2^-$ and H_2O_2 (perhaps as a result of electron leakage from the photosynthetic and respiratory electron transport chains to oxygen).

H_2O_2 is generated with various environmental and developmental stimuli; its accumulation could reflect the oxidative stress and the changes of antioxidants in different compartments of the plant. In our results, it seems that ROS, including superoxide and H_2O_2, are elevated with increased salinity, due to the imbalance in the production and destruction of ROS (Asada, 1994; Ellouzi et al., 2011). Furthermore, SA, AsA, Pro and GSH have been found to provide a protective action against salt-induced oxidative damage in various plants through the enhancement of multiple processes including growth components, enhanced Pro accumulation and decreased H_2O_2 content (Gunes et al., 2007; Younis et al., 2009 and 2010; Hossain and Fujta, 2010; Azzedine et al., 2011; Younis and Tourky, 2014).

Changes in lipid peroxidation and membrane injury

In relation to control levels, lipid peroxidation in both sensitive and tolerant broad bean seedlings, salinized with low and high levels of NaCl, showed a significant increase after 14 days of germination; this positive response being more pronounced with the high salinity level. Also lipid peroxidation, as determined by MDA content, in the salt-tolerant cultivar was consistently lower than that in the salt-sensitive one (Table 1).

Treatment of both cultivars with Pro, AsA, GSH or SA adaptive compound appeared to decrease lipid peroxidation below those control contents; the magnitude of decrease being more or less comparable in both cultivars of broad beans. However, SA treatment appeared to be the most inductive in reduction of lipid peroxidation. In both cultivars of bean seedlings, exogenous application of each of the adaptive compounds with each level of NaCl, partially reduced the increments in lipid peroxidation maintained in response to treatment with each of the two levels of NaCl alone (Table 1).

The pattern of changes in membrane injury, being determined as electrolyte leakage, from the variously treated broad bean cultivars, appeared more or less comparable with those changes maintained for lipid peroxidation (Table 1) which has been repeatedly reported to cause peroxidative damage to plasma membranes (Hanaa et al., 2003; Koca et al., 2007).

Determining the MDA content and hence, the extent of membrane lipid peroxidation, has often been used as a tool to assess the degree of plant sensitivity to oxidative damage (Blokhina et al., 2003). Our results lend a strong support to that of Koca et al. (2007) which showed that lipid peroxidation was higher at 100 mM NaCl treatment in salt-sensitive cultivar of *Sesamum indicum* than in the salt-tolerant one. Also, Hanaa et al. (2003), experimenting with three onion cultivars salinized with 2000, 4000 and 6000 ppm salts prepared from seawater, found a progressively greater increase in lipid peroxidation with an increase in salt levels, and it was suggested that the increase in lipid peroxidation may be due to incapability of antioxidants to neutralize and scavenge all the ROS resulting from salt stress.

As lipid peroxidation induces a peroxidative damage to plasma membranes (Mittler, 2002; Apel and Hirt, 2004; Lee et al., 2004; Parida and Das, 2005), it is thus expected to induce electrolyte leakage. That increased lipid peroxidation is associated with electrolyte leakage is now confirmed (see table 1 in addition to e.g. Shalata and Neumann (2001) using tomato and Bandeoglu et al. (2004) using lentil).

Furthermore, as a consequence of salinity stress, lipid peroxidation in maize (Gunes et al., 2007), tomato (Shalata and Neumann, 2001), tobacco (Okuma et al., 2004) and canola (Khattab, 2007) plants was decreased by exogenous application of SA, AsA, Pro and GSH, respectively. Using broad beans, our results presented in table 1 well support these observations and further indicate that both the amounts of MDA and ion leakage from salt-treated seedlings being variably reduced by the use of the four adaptive compounds. Thus, it is now confirmed that additional adaptive compounds might inhibit the peroxidative damage to plasma membranes and SA appeared to be the most effective in this action. Possibly, the protective effect of the adaptive compounds is

Table 1. Effects of salinity alone or in combination with proline, ascorbic acid, reduced glutathione or salicylic acid on the hydrogen peroxide content, lipid peroxidation and electrolyte leakage of salt-sensitive and salt-tolerant broad beans after 14 days of germination.

Treatments	Giza 716 (salt-sensitive)			Giza 843 (salt-tolerant)		
	H_2O_2 (mmole g^{-1} fresh weight)	Lipid Peroxidation (mmole MDA g^{-1} fresh weight)	Electrolyte Leakage (% ion leakage)	H_2O_2 (mmole g^{-1} fresh weight)	Lipid Peroxidation (mmole MDA g^{-1} fresh weight)	Electrolyte Leakage (% ion leakage)
Control (1/10 Hoagland soln.)	32.33	0.53	66.14	31.26	0.42	45.03
50 mM NaCl	47.92*	0.60*	80.22*	48.55*	0.47*	52.26*
300 mM NaCl	68.66*	0.75*	94.58*	56.44*	0.58*	62.56*
0.40 mM Pro	32.56*	0.52*	65.62*	30.89*	0.42*	44.92*
50 mM NaCl + 0.40 mM Pro	45.82*	0.67*	73.77*	42.93*	0.50*	49.22*
300 mM NaCl + 0.40 mM Pro	56.04*	0.67*	77.20*	53.62*	0.52*	52.11*
4.0 mM AsA	31.11*	0.52*	65.51*	30.14*	0.41*	44.82*
50 mM NaCl + 4.0 mM AsA	43.63*	0.65*	73.62*	40.61*	0.49*	48.66*
300 mM NaCl + 4.0 mM AsA	56.62*	0.66*	77.11*	51.42*	0.52*	51.22*
1.0 Mm GSH	34.32*	0.52*	66.00*	31.14*	0.42*	44.96*
50 mM NaCl + 1.0 mM GSH	47.21*	0.67*	74.21*	45.33*	0.50*	50.50*
300 mM NaCl + 1.0 mM GSH	57.70*	0.70*	78.03*	54.33*	0.52*	52.40*
0.09 mM SA	30.22*	0.52*	65.30*	29.23*	0.41*	44.63*
50 mM NaCl + 0.09 mM SA	40.24*	0.63*	72.55*	38.66*	0.46*	48.02*
300 mM NaCl + 0.09 mM SA	52.66*	0.65*	76.00*	50.92*	0.51*	51.14*

*The mean values are significantly different from the control at $P \leq 0.05$.

Table 2. Effects of salinity alone or in combination with proline, ascorbic acid, reduced glutathione or salicylic acid on total phenolics, anthocyanin, ascorbic acid and reduced glutathione of salt-sensitive and salt-tolerant broad beans after 14 days of germination.

Treatments	Giza 716 (salt-sensitive)				Giza 843 (salt-tolerant)			
	Total Phenolics (mg catechol equivalent g^{-1} dry weight)	Anthocyanin (mg anthocyanin g^{-1} dry weight)	AsA (mmole g^{-1} fresh weight)	GSH (mmole g^{-1} fresh weight)	Total Phenolics (mg catechol equivalent g^{-1} dry weight)	Anthocyanin (mg anthocyanin g^{-1} dry weight)	AsA (mmole g^{-1} fresh weight)	GSH (mmole g^{-1} fresh weight)
Control (1/10 Hoagland soln.)	0.82	0.43	25.18	14.18	0.90	0.48	34.83	20.55
50 mM NaCl	0.89*	0.41*	28.72*	16.95*	0.99*	0.46*	38.22*	26.03*
300 mM NaCl	1.03*	0.35*	35.68*	19.33*	1.14*	0.42*	43.67*	37.66*
0.40 mM Pro	0.93*	0.52*	26.48*	14.96*	1.19*	0.65*	30.08*	20.94*
50 mM NaCl + 0.40 mM Pro	1.06*	0.47*	30.96*	21.74*	1.25*	0.65*	40.71*	42.41*
300 mM NaCl + 0.40 mM Pro	1.16*	0.37*	42.22*	39.82*	1.30*	0.46*	52.69*	48.56*
4.0 mM AsA	0.95*	0.54*	26.86*	15.22*	1.24*	0.65*	30.93*	22.67*
50 mM NaCl + 4.0 mM AsA	1.09*	0.49*	34.66*	21.92*	1.29*	0.66*	44.32*	45.21*
300 mM NaCl + 4.0 mM AsA	1.19*	0.37*	46.24*	41.46*	1.38*	0.46*	55.33*	50.26*
1.0 mM GSH	0.93*	0.52*	25.70*	14.69*	1.19*	0.65*	29.75*	20.60*
50 mM NaCl + 1.0 mM GSH	1.05*	0.46*	30.55*	21.11*	1.25*	0.64*	40.22*	40.57*
300 mM NaCl + 1.0 mM GSH	1.13*	0.36*	40.51*	37.59*	1.30*	0.45*	52.21*	46.99*
0.09 mM SA	0.97*	0.55*	27.00*	15.33*	1.25*	0.67*	31.87*	24.16*
50 mM NaCl + 0.09 mM SA	1.11*	0.51*	39.72*	22.88*	1.31*	0.67*	48.79*	48.48*
300 mM NaCl + 0.09 mM SA	1.21*	0.40*	50.11*	46.52*	1.40*	0.47*	56.29*	53.53*

*The mean values are significantly different from the control at $P \leq 0.05$.

Table 3. Effects of salinity alone or in combination with proline, ascorbic acid, reduced glutathione or salicylic acid on antioxidant enzymes (SOD, CAT, APX, GR, POX and PPO) of salt-sensitive and salt-tolerant broad beans after 14 days of germination.

Treatments	Giza 716 (salt-sensitive)					
	SOD U mg^{-1} (protein) s^{-1}	CAT mmole red H_2O_2 mg^{-1} (protein) s^{-1}	APX mmole ascorbic acid mg^{-1} (protein) s^{-1}	GR mmole red GSH mg^{-1} (protein) s^{-1}	POX U g^{-1} fresh weight min^{-1}	PPO U g^{-1} fresh weight min^{-1}
Control (1/10 Hoagland soln.)	19.56	22.72	9.56	7.56	52.35	15.00
50 mM NaCl	24.22*	26.90*	14.83*	11.42*	76.66*	17.70*
300 mM NaCl	14.15*	20.22*	12.42*	9.37*	40.15*	3.80*
0.40 mM Pro	26.62*	23.09*	10.15*	9.12*	80.16*	17.33*
50 mM NaCl + 0.40 mM Pro	29.12*	42.55*	21.78*	25.65*	110.14*	18.71*
300 mM NaCl + 0.40 mM Pro	25.23*	30.18*	16.69*	20.22*	107.91*	19.32*
4.0 mM AsA	28.09*	23.81*	10.56*	9.87*	93.29*	18.21*
50 mM NaCl + 4.0 mM AsA	30.68*	44.21*	25.24*	26.44*	112.47*	24.10*
300 mM NaCl + 4.0 mM AsA	25.78*	32.26*	17.84*	20.25*	112.36*	21.73*
1.0 mM GSH	25.72*	22.92*	9.88*	8.58*	78.71*	16.96*
50 mM NaCl + 1.0 mM GSH	20.93*	38.35*	20.55*	24.22*	109.40*	19.66*
300 mM NaCl + 1.0 mM GSH	21.89*	29.94*	16.26*	19.14*	86.15*	18.66*
0.09 mM SA	30.99*	25.63*	10.60*	10.18*	108.32*	19.96*
50 mM NaCl + 0.09 mM SA	33.52*	45.30*	27.43*	26.97*	120.54*	24.21*
300 mM NaCl + 0.09 mM SA	26.81*	36.35*	19.33*	21.13*	114.28*	22.92*

Treatments	Giza 843 (salt-tolerant)					
	SOD U mg^{-1} (protein) s^{-1}	CAT mmole red H_2O_2 mg^{-1} (protein) s^{-1}	APX mmole ascorbic acid mg^{-1} (protein) s^{-1}	GR mmole red GSH mg^{-1} (protein) s^{-1}	POX U g^{-1} fresh weight min^{-1}	PPO U g^{-1} fresh weight min^{-1}
Control (1/10 Hoagland soln.)	22.42	30.50	11.30	9.88	77.02	16.92
50 mM NaCl	28.55*	34.62*	18.66*	15.26*	81.62*	20.24*
300 mM NaCl	18.29*	28.27*	14.26*	11.16*	50.25*	8.45*
0.40 mM Pro	31.26*	32.36*	12.07*	12.93*	100.53*	20.61*
50 mM NaCl + 0.40 mM Pro	52.24*	44.22*	29.69*	26.14*	110.24*	29.71*
300 mM NaCl + 0.40 mM Pro	40.83*	37.82*	22.45*	21.78*	108.73*	21.59*
4.0 mM AsA	32.66*	34.60*	12.85*	13.46*	107.31*	22.00*
50 mM NaCl + 4.0 mM AsA	53.62*	47.88*	31.18*	29.77*	121.35*	29.59*
300 mM NaCl + 4.0 mM AsA	42.59*	39.22*	26.96*	23.12*	111.95*	25.32*
1.0 mM GSH	28.98*	32.22*	11.73*	12.40*	94.70*	19.26*
50 mM NaCl + 1.0 mM GSH	46.30*	41.46*	27.25*	25.48*	109.19*	28.14*
300 mM NaCl + 1.0 mM GSH	38.46*	34.14*	20.83*	18.62*	96.22*	20.93*
0.09 mM SA	34.14*	35.67*	13.22*	14.62*	116.12*	24.46*
50 mM NaCl + 0.09 mM SA	57.37*	51.93*	32.39*	30.72*	129.65*	30.66*
300 mM NaCl + 0.09 mM SA	45.90*	40.51*	28.72*	23.92*	121.84*	27.55*

*The mean values are significantly different from the control at $P \leq 0.05$.

related to reduced ROS damage to essential proteins and/or nucleic acids and/or its binding to the membrane lipids may stabilize the membrane permeability structure, and so maintain its properties and functions.

Changes in antioxidant system

Plant phenolics

Treatment of both sensitive and tolerant broad beans with NaCl induced a significant increase in plenolic compounds in contrast with a significant decrease in anthocyanins; the magnitude of response being more pronounced with high NaCl concentration. Also the absolute values of total phenol contents as well as of anthocyanin contents were consistently higher in the tolerant seedlings than those contents obtained in the sensitive ones (Table 2). Supplemental treatment with Pro, AsA, GSH or SA, induced a significant increase in total phenols, as well as in anthocyanin contents, after 14 days of germination; the magnitude of increase above the control values being most pronounced with SA and also appeared more operative in the salt-tolerant cultivar (Table 2).

In both cultivars, combination of salinity levels with Pro, AsA, GSH or SA induced a significant increase in total phenolics as well as a significant decrease in anthocyanin contents after 14 days of germination in the respective culture media, as compared with each of the adaptive compounds when used alone, the magnitude of response appeared more operative in the tolerant cultivar. In the various samples treated with the adaptive compounds, the magnitude of the maintained changes (increase in phenolic compounds and decrease in anthocyanins) were most pronounced upon the use of SA followed by AsA and Pro. GSH, however, appeared to be the least in its response.

Ascorbic acid and reduced glutathione

As indicated in table 2, the contents of AsA and GSH in the tolerant cultivar were consistently higher than those contents in the sensitive one. As compared with control levels, salinization of both cultivars induced a significant increase in the contents of AsA and GSH after 14 days of germination, the magnitude of response was more pronounced with 300 mM NaCl.

In relation to control values, treatment with each of the adaptive compounds induced a significant slight increase in AsA and GSH contents in both cultivars, except for that of AsA which showed a significant decrease in the salt-tolerant cultivar. The following sequence of treatments (SA > AsA > Pro > GSH > control) was, in general, displayed with respect to the increase maintained in contents of AsA and GSH in both cultivars. On the other hand, combination of salinity with each of the adaptive compounds induced a significant increase in the contents of AsA and GSH in both cultivars as compared with salinized controls. The increased levels appeared most pronounced with the high level of salinity and with SA (Table 2).

Antioxidant enzymes

Perusal of the data presented in table 3 led us to point out that, in relation to control levels, the activities of SOD, CAT, APX, GR, POX and PPO in both sensitive and tolerant beans, salinized with 50 mM NaCl, showed a marked significant increase. On the other hand, 300 mM NaCl induced a significant decrease in the activities of SOD, CAT, POX and PPO below the control levels, whereas those activities of APX and GR were found to be increased. For all these investigated enzymes, the activities were markedly higher in the tolerant cultivar than in the sensitive one.

For both cultivars of beans, treatment with Pro, AsA, GSH or SA induced an increase in the activities of all the investigated enzymes. Upon combination of each of the salinity levels with Pro, AsA, GSH or SA, the apparent general response was a marked increase in the activities of all enzymes above the control levels detected in samples treated solely with Pro, AsA, GSH or SA. The magnitude of the positive response was higher when the adaptive compounds were combined with the low NaCl and the following sequence of treatments: SA > AsA > Pro > GSH, was displayed with respect to the magnitude of increase in activities of all enzymes investigated (Table 3).

Under normal conditions, the production and destruction of ROS is well regulated in plant cells, but when plants are subjected to stress, the balance between the production of ROS and the quenching activity of the antioxidant system is upset, often resulting in oxidative stress (Apel and Hirt, 2004; Parida and Das, 2005). Plants with high levels of antioxidants, mainly plant phenolics, AsA and GSH have been reported to have greater resistance to this oxidative damage (Gould et al., 2000; Zheng and Wang, 2001; Noctor et al., 2002; Mittova et al., 2003; Younis et al., 2010). Thus, perusal of our data (Table 2) indicate that plant phenolics including anthocyanins as well as AsA and GSH play an important role as antioxidants in broad seedlings, under the present salt stress conditions. This is mainly because of the increased internal effective concentrations of these antioxidant compounds in the salt-tolerant cultivar, due to acquired varietal adaptation to salinity tolerance, as well as to exogenous application of AsA, GSH, SA and Pro to both cultivars of broad beans. These adaptive compounds are also known to reorganize the balance between the constitutive antioxidant compounds and enzymes of the defense system in plant tissues in a way that seems to be directly involved in ROS detoxification (Zheng and Wang, 2001; Athar et al., 2008; Younis et al., 2010).

To overcome the effects of salinity-induced stress, plants make use of a complex antioxidant system. Thus, tolerance to NaCl stress in higher plants correlates to the levels of antioxidant enzymes and substrates (Koca et al., 2007; Athar et al., 2008). Also, this has become evident from various investigations since relatively higher activities of ROS-scavencing enzymes are reported in tolerant genotypes when compared to the susceptible ones (Reddy et al., 2004; Nawaz and Ashraf, 2007). The results obtained in the present study using two cultivars of broad beans seedlings, contrasting in salt tolerance (Table 3), well support these conclusions. It is also evident that each of the scavenging enzymes detected in this study has a specific role in overcoming the oxidative stress induced by salinity.

Our results (Table 3) greatly indicate that the used adaptive compounds profoundly activate the defense system in order to alleviate oxidative damage induced by salt stress in both bean cultivars. In support of our results, SA when supplied exogenously, it was found to enhance the efficiency of the enzymatic antioxidant system in plants (Yusuf et al., 2008; Noreen et al., 2009). Thus, it is evident that the antioxidant system ontribute substantially to SA-induced adaptation to subsequent stress conditions. Also, application of AsA increased the activity of antioxidant enzymes and the content of endogenous AsA. This may reduce the accumulation of ROS in salt stress seedlings (Athar et al., 2008). As became evident also from Younis et al., 2010 study, AsA and GSH in association with the activities of SOD, APX, CAT and GR

enzymes seemed to be directly involved in ROS detoxification.

Exogenous application of Pro was found to alleviate the growth inhibition of cucumber plants induced by NaCl and to decrease cellular H_2O_2 content, MDA and electrolyte leakage, while cellular concentration of Pro was increased (Huang et al., 2009). Furthermore, Khattab (2007) using salt-sensitive and salt-tolerant canola plants found that exogenously applied GSH enhanced the activity of SOD in plants of the two cultivars.

As have been made clear in our previous communication (Younis and Tourky, 2014), the salt-tolerant cultivar being already acclimated to salt tolerance by accumulation of osmolytes (Younis and Tourky, 2014) also maintained contents and activities of the antioxidant system that appeared higher than those present in the salt-sensitive cultivar. As already stated, this can be considered as acquired varietal adaptation to salinity tolerance in broad bean plants.

Materials and Methods

Plant material

Two homogeneous lots of broad beans: *Vicia faba* var. Giza 716 (salt-sensitive cultivar) and Giza 843 (salt-tolerant cultivar) were obtained from Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt.

Determination of hydrogen peroxide

The contents of H_2O_2 were measured according to Velikova et al. (2000).

Determination of lipid peroxidation

According to Heath and Packer (1968), MDA content was measured and calculated by using extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as mmol MDA g^{-1} fresh weight.

Determination of membrane injury

Fresh seedlings were cut into 5 mm length sections and placed in a test tube containing 10 cm^3 distilled water. The tubes were covered and maintained at constant temperature of 30 ^oC. After 2 h, initial electrical conductivity of the medium (EC1) was measured. The samples were autoclaved at 120 ^oC for 20 min to completely kill the tissue and release all electrolytes. After cooling at 25 ^oC, the final electrical conductivity (EC2) was measured. The membrane injury index herein determined as electrolyte leakage (EL) was expressed following the formula EL= EC1 / EC2 × 100 (Dionisio-Sese and Tobita, 1998).

Estimation of antioxidant compounds

a- **Plant phenolics** were determined as described by Malik and Singh (1980). Total phenols were extracted from dried powdered bean seedlings and estimated using Folin-Ciocalteau reagent. The optical density of the colour developed was measured at 650 nm. The total phenolics were calculated as mg catechol equivalent g^{-1} dry mass. Anthocyanins were also extracted from dried powdered tissues and determined using the method of Mirecki and Teramura (1984).

b- **Reduced glutathione** in fresh seedlings was extracted and assayed according to Cao *et al.* (2004).

c- **Ascorbic acid** content was estimated in homogenized fresh bean seedlings as indicated by Law *et al*. (1983).

Estimation of antioxidant enzyme activities

Enzyme extraction. Fresh seedlings (0.2 g) were homogenized in a mortar with 5 cm^3 of chilled phosphate buffer. For APX and SOD, the extraction medium was 0.1 M phosphate buffer at pH 7.8 and for CAT, GR, POX and PPO, 0.1 M phosphate buffer at pH 6.8 was used. The homogenate was filtered and the filtrate centrifuged at 10,000 g for 20 min. The supernatant served as enzyme extract (Agarwal and Shaheen, 2007).

a- Superoxide dismutase. According to Giannopolitis and Ries (1977), one enzyme unit of SOD activity is defined as the amount of enzyme required to cause 50 % inhibition of the rate of nitro-blue-tetrazolium (NBT) reduction measured at 560 nm.

b- Catalase. Activity was determined by measuring changes in absorbance at 510 nm corresponding to the decomposition of H_2O_2 in a reaction mixture as adopted by Aebi (1984).

c- Ascorbate peroxidase. Activity was assayed by measuring the decrease in absorbance at 290 nm due to ascorbate oxidation; as described by Nakano and Asada (1981).

d- Glutathione reductase. Aactivity was assayed according to the method adopted by Goldberg and Spooner (1983).

e- Peroxidase. Activity was assayed as the increase in absorbance at 420 nm due to the formation of purpurogallin (Devi, 2002).

f- Polyphenol oxidase. Activity was assayed as the increase in absorbance at 420 nm due to the formation of purpurogallin (Devi, 2002).

Time course experiments

The experimental plan, treatment and sampling procedures were essentially the same as adopted by Younis and Tourky (2014). Seeds of two homogeneous groups of broad beans: *Vicia faba* var. Giza 716 (salt-sensitive cultivar) and Giza 843 (salt-tolerant cultivar) were selected, surface sterilized and thoroughly washed with distilled water. Thereafter, each group of the sterilized seeds was divided into 15 subgroups that were primed separately in aerated solutions of different concentrations of either NaCl (50 and 300 mM) or an optimum concentration of each of the used adaptive compounds (Younis and Tourky, 2014) maintained in 1/10 Hoagland solution for 24 h at room temperature. Thus, for each cultivar of the broad beans used, a total of 15 treatments representing all planned possible combinations of salinity levels and adaptive compounds were allotted according to the following treatment scheme:

1- 1/10 Hoagland solution (control)	7- 4.0 mM AsA
2-50 mM NaCl	8- 50 mM NaCl + 4.0 mM AsA
3-300 mM NaCl	9- 300 mM NaCl + 4.0 mM AsA
4- 0.40 mM Pro	10- GSH 1.0 mM
5- 50 mM NaCl + 0.40 mM Pro	11- 50 mM NaCl + 1.0 mM GSH
6- 300 mM NaCl + 0.40 mM Pro	12-300 mM NaCl + 1.0 mM GSH
	13-0.09 mM SA
	14- 50 mM NaCl + 0.09 mM SA
	15- 300 mM NaCl + 0.09 mM SA

The subgroup of seeds allotted for each treatment was subdivided into a number of sets that were allowed to germinate in plastic boxes furnished with chromatographic paper (Whatman No.1) moistened by 1/10 Hoagland solution (for control samples) and NaCl or adaptive compound; each at an appropriate concentration maintained in 1/10 Hoagland nutrient solution. The germination boxes were incubated in the dark at 20 ± 0.1°C. During the experimental period of 14 days, when required, each box was supplied with equal additional amounts of the appropriate solutions.

Seeds were examined every other day under sterile conditions and on the 14th day, triplicate samples of seedlings were immediately decoated, dried on paper towels and then subjected to chemical and enzymatic analyses. The obtained data were statistically analyzed using one way analysis of variance (ANOVA) and comparison among means was carried out by calculating L.S.D. with a significant level at $P \leq 0.05$.

Conclusions

Combination of the various adaptive compounds with low and high salinity levels led to different rates of counteraction of the adverse effects of salinity in both cultivars; the magnitude of response being higher in the tolerant than in the sensitive cultivar. Nevertheless, the following sequence of treatments: SA > AsA > Pro > GSH was obtained.

During the past few decades, intensive studies were carried out by a large number of investigators in an attempt to explore salinity tolerance in a vast group of plant species. Today, there is increasing use of saline water in agriculture in arid and in semiarid regions. Though promising results were obtained, yet we have long way of experimentation to go in an attempt to adapt plants to grow better under salt stress conditions. As leguminous plants represent a major group of important crop plants in large investigated areas of Egypt, information of their responses to different stresses is vital. From our results herein described as well as those published by Younis and Tourky (2014), the possibility of plantation of faba bean, in particular the salt-tolerant cultivar, in newly reclaimed areas, in which the use of saline water or even diluted sea water are the only available source of irrigation, is suggested.

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