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Exogenous application of proline alleviates salt-induced oxidative stress in *Phaseolus vulgaris* L. plants

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SUMMARY

Two field experiments on common bean (*Phaseolus vulgaris* L.) plants were conducted at three sites having different levels of salinity (EC = 1.84, 6.03, or 8.97 dS m^{-1}) and considered to be low, moderate, or highly saline soil, respectively. The aim was to examine the effects of three successive exogenous applications of 5.0 mM proline, applied as foliar sprays at 20, 30, and 40 d after sowing (DAS) to each plant at each site. Bean plants were sampled 50 DAS and the effects of the proline sprays on various growth parameters, levels of photosynthetic pigments, endogenous proline, ascorbic acid, nitrate, nitrite, and mineral nutrient (P, K, Na) concentrations, and anti-oxidant enzyme activities were measured in order to understand the mechanism(s) of salt tolerance in proline-treated bean plants. Exogenous applications of 5 mM proline alleviated oxidative stress and enhanced the growth of all treated common bean plants. Proline also increased the activities of the anti-oxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), as well as the concentrations of carotenoids, ascorbic acid, and endogenous proline. Spray applications of proline increased the concentrations of P and K^+, and decreased Na^+ ion concentrations, in salt-affected plants. Thus, the K^+:Na^+ ratio increased. Based on these findings, we recommend the use of proline as a commercial formulation to enhance plant growth and production in common bean plants grown under saline conditions.

Salinity is one of the most serious abiotic stress factors that limit crop productivity. Salinity affects plant physiology at both the whole plant and cellular levels by causing osmotic and ionic stresses. Salinity generates physiological drought, or osmotic stress, by affecting the water relations of the plant (Munns, 2002). Photosynthesis is one of the most severely affected processes during salinity stress (Sudhir and Murthy, 2004), which is caused by decreased levels of chlorophyll and inhibition of the key photosynthetic enzyme, Rubisco (Soussi *et al.,* 1998). This and other affected processes lead to poor plant growth and low productivity. A common consequence of most abiotic stresses, including salinity, is the increased production of reactive oxygen species (ROS) such as superoxide radicals ($O_{2^{\cdot-}}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}HO$) which are extremely toxic to plants and cause damage to DNA, proteins, lipids, and chlorophyll (Halliwell and Gutteridge, 2000). However, plants can respond with anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), and with non-enzymatic anti-oxidants such as ascorbic acid, glutathione, and carotenoids to counteract this oxidative stress and to protect themselves from oxidative damage (Apel and Hirt, 2004).

Over the last few decades, in parallel with traditional breeding and biotechnological strategies to improve the tolerance of plants to salinity (Maggio *et al.,* 2003), several techniques have been proposed to improve plant performance in saline environments. These include seed or seedling priming (Azooz, 2009), pre-exposure to moderate salt stress (Friedman *et al.,* 2006), and/or the application of stress metabolites that could be recognised and integrated by plants as components of a stress-induced adaptation response (Ashraf and Foolad, 2007). Foliar applications of osmo-protective molecules such as proline (Pro) have also been shown to have beneficial effects on plants exposed to salt stress (Ali *et al.,* 2007). The mechanism(s) by which these molecules exert their protective function is (are) not fully understood. It is probable that they accumulate in the cytoplasm and assist in the osmotic adjustment which is necessary to overcome hyper-osmotic stress (Yoshiba *et al.,* 1997). Alternatively, the osmoprotectants may act as stress signals that elicit a series of protective responses, including the accumulation of anti-oxidants and other active metabolites that contribute to the adaptation to stress (Maggio *et al.,* 2002). While applications of proline have been shown to improve yields in salt-stressed crops (Ashraf and Foolad, 2007), their effects on the quality parameters of commercial products are largely unknown. Nevertheless, the link between adaptation to stress and the accumulation of high-value metabolites may be important for agricultural production (De Pascale *et al*., 2001).

This study was conducted to investigate the effects of exogenous applications of proline on various growth parameters, on the concentrations of endogenous

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proline, photosynthetic pigments, ascorbic acid, nitrate, nitrite, and mineral nutrients, and on anti-oxidant enzyme activities in common bean plants grown under three levels of saline stress. The aim was to improve our understanding of the mechanisms of salt tolerance in proline-treated plants.

MATERIALS AND METHODS

Plant material and experimental procedures

Two field experiments were conducted in two successive seasons (2010 and 2011) in Experimental Farm of the Faculty of Agriculture, Fayoum University, Southeast Fayoum, Egypt (29º 17'N; 30º 53'E). In the 2010 season, daily temperatures ranged from 14.1º -26.9ºC, with an average of 20.5º ± 2.7ºC. The daily relative humidity averaged 54.0 ± 4.6%, and ranged from 22 - 83%. In the 2011 season, daily temperatures ranged from 9.4º - 27.8ºC, with an average of 18.2º ± 2.9ºC, and the daily relative humidity averaged 54 ± 7.3% and ranged from 20 - 84%.

Healthy common bean (*Phaseolus vulgaris* L.) cv. Bronco seeds were sown on 25 February 2010, and on 20 February 2011. Seeds were obtained from The Horticulture Research Institute, The Agricultural Research Centre, Giza, Egypt, and were sown at the equivalent of 95 kg ha^{-1} to achieve the recommended planting density.

Common bean seeds were selected for uniformity by choosing those of equal size and of the same colour. The selected seeds were washed with distilled water, sterilised in 1% (v/v) sodium hypochlorite for approx. 2 min, washed thoroughly again with distilled water, and left to dry at room temperature (25ºC) for approx. 1 h. Uniform, air-dried common bean seeds were sown in hills in rows were spaced 10 – 15 cm apart in plots (5 rows; 3m long and 60cm width). Thinning was done before the first irrigation to produce two plants per hill. During soil preparation and plant growth, the soil was supplemented with the full dose of NPK fertiliser according to the recommendations of the Ministry of Agriculture and Land Reclamation for the area studied. These recommendations were for 475 $kgha^{-1}$ of calcium super-phosphate (15.5% P_2O_5), 120 kg ha^{-1} ammonium sulphate (20.5 %N), and 60 kg ha^{-1} potassium sulphate (48% K_2O) during seed-bed preparation. An additional 120 kg ha^{-1} of ammonium sulphate and 60 kg ha^{-1} of potassium sulphate were added at 3 and at 6 weeks after each sowing. Irrigation water was added to 100% of the reference crop evapotranspiration (ETo), values from the Fayoum Meteo Station. Seven irrigations were applied in each season, with total water rates of 2,750.4 m^3 ha^{-1} and 2,829.6 m^3 ha^{-1} in 2010 and 2011, respectively. All other recommended agricultural practices were followed.

Three experimental sites were chosen in each season. Soil analyses were carried out according to Black *et al*. (1965) and Jackson (1973). The results from physical and chemical analyses of the soils are shown in Table I. Electrical conductivity (EC) was measured using a conductivity meter and an extract of each soil paste. Soil EC values were 1.84, 6.03, and 8.97 dS m^{-1} at sites 1, 2, and 3, respectively. These EC values classed the soils as being non-saline, moderately saline, or strongly saline at sites 1, 2, and 3, respectively, according to Dahnke and Whitney (1988).

Twenty days after sowing (DAS), seedlings in each plot were sprayed to run-off with 5.0 mM proline or with tap water as a control (0 mM proline). Proline applications were repeated at 30 and 40 DAS. The concentration of proline (Sigma-Aldrich Co. Taufkirchen, Germany), and the number and timing of sprays were based on results from a preliminary pot trial (data not shown). To ensure optimal penetration into leaf tissues, 0.1% (v/v) Tween-20 was added to the foliar sprays as a surfactant.

The experiment was arranged in a randomised complete block design, with two levels of Pro (0 or 5.0 mM), at three sites differing in soil salinity, with three replicate plots per site.

Measurement of chlorophyll, caroteniod, proline and ascorbic acid concentrations

Plant samples were collected 50 DAS in both seasons. In each season, 108 plants (i.e., six plants from each plot × two levels of proline spray × three soil salinity levels × three replicate plots) were carefully removed from the experimental site and dipped in a bucket of water. Plants

TABLE I
Mechanical and chemical analyses of the soils used at the three experimental sites

Properties	Site 1	Site 2	Site 3
Mechanical analyses			
Coarse sand [%(w/v)]	3.15	3.75	2.85
Fine sand [%(w/v)]	63.85	65.25	47.15
Silt [%(w/v)]	19.75	20.25	20.50
Clay [%(w/v)]	13.25	10.75	29.50
Soil texture	Sand, loamy	Sand, loamy	Sand clay, loamy
pH [at a soil:water (w/v) ratio of 1:2.5]	7.36	7.64	7.81
EC (dS m^{-1}; soil-paste extract)	1.84	6.03	8.97
Chemical analyses			
Organic matter [%(w/v)]	1.33	1.12	1.01
$CaCO_3$ [%(w/v)]	8.14	7.86	6.95
Total N [%(w/v)]	0.08	0.07	0.06
Available nutrients (mg kg^{-1} soil)			
P	6.33	7.21	8.22
K	210.9	198.1	181.15
Fe	7.03	6.94	5.72
Mn	2.48	1.04	0.98
Zn	0.78	0.75	0.66
Cu	0.46	0.42	0.33

were shaken gently to remove all adhering soil particles and the lengths of their roots and shoots were measured. To determine total leaf chlorophyll, carotenoids, and ascorbic acid concentrations, 2 g of fresh leaf tissue were used. The remainder of each plant, was then oven-dried for 72 h at 70ºC, and its dry weight (DW) was recorded.

Leaf total chlorophyll and carotenoids concentrations [in mg g^{-1} fresh weight (FW)] were estimated using 80% (v/v) acetone extracts and the spectrophotometric method according to Lichtenthaler and Wellburn (1983).

Proline (Pro) contents (in µg g^{-1} DW of leaf) were measured using the rapid colourimetric method suggested by Bates *et al*. (1973). Proline was extracted from 0.5 g DW of leaf tissue by grinding in 10 ml of 3% (v/v) sulphosalicylic acid. The mixture was then centrifuged at 10,000 × g for 10 min. Two ml of the supernatant was placed in a test tube and 2 ml of freshly prepared acid-ninhydrin solution was added. The tubes were incubated in a water bath at 90ºC for 30 min and the reaction was terminated in an ice-bath. Each reaction mixture was then extracted with 5 ml of toluene and vortex-mixed for 15 s. The tubes were allowed to stand for at least 20 min in the dark at room temperature to separate the toluene and aqueous phases. The toluene phase was then collected carefully into a test tube and its absorbance was read at 520 nm. The concentration of Pro in each sample was determined from a standard curve prepared using analytical grade Pro and expressed on a 100 g^{-1} DW basis.

The extraction and determination of the ascorbic acid (AsA) contents of leaves (in mg 100 g^{-1} FW) were carried out following the method of Kampfenkel *et al*. (1995). Each sample of leaf material (1.0 g) was homogenised rapidly in liquid N_2 and extracted with 10 ml of 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4ºC for 5 min at 15,600 × g. The supernatant was transferred to a fresh reaction vessel and assayed immediately for its ASA content in a reaction mixture containing, 10 mM DTT, 0.2 M phosphate buffer (pH 7.4), 0.5% (w/v) N-ethyl maleimide, 10% (w/v) TCA, 42% (w/v) H_3PO, 4% (v/v) 2,2'-dipyridyl, and 3% (w/v) $FeCl_3$.

Nitrate and nitrite concentrations in leaves

Samples were prepared by washing leaves in tap water, then several times in distilled water. Leaves were cut into uniformly-sized pieces (2.0 cm^2) to facilitate oven-drying at the same rate. The samples were dried in an oven at 105ºC for 24 h until they were brittle and crisp. At this stage, no microorganisms had grown and care was taken to avoid any such contamination. The dried samples were ground using a clean mortar and pestle, and sieved to obtain a < 2.0 mm size-fraction. A portion (1.0 g) of each sieved leaf sample was placed in a 100 ml glass bottle and 40 ml of distilled water was added. The bottle was capped and shaken for 30 min, then the mixture was filtered and the filtrate was made-up to 100 ml in a volumetric flask (Radojevic and Bashkin, 1999). Measurements of the nitrate ion (NO_3^-) concentration of each leaf extract was done using a spectrophotometer (Model 2000; Kwf Sci-Tech Development Co. Ltd., Beijing, P. R. China) at 543 nm. The pre-programme for NO_3^- (64 NO_3^--N) was selected and readings were converted to NO_3^- concentrations (mg g^{-1} DW leaf) by multiplying with a conversion factor of 4.4 (LaMotte, 2000). The NO_3^- ion concentration (in mg g^{-1} DW) of each leaf sample was calculated using the formula:

$$NO_3^- \text{ content (in mg } g^{-1} \text{ DW)} = \frac{C \times V}{M}$$

where, C was the concentration of NO_3^- in the sample (in mg g^{-1} DW), V was the total volume of the sample solution (100 ml), and M was the DW of the sample (1.0 g; LaMotte, 2000).

Nitrite (NO_2^-) ion concentrations were determined in a similar manner. The pre-programme number for NO_2^- was 67 NO_2^--N, and the reaction time was 5 min compared to 10 min for NO_3^- ions. NO_2^--N contents were converted to mg g^{-1} DW leaf tissue by multiplying by 3.3 (LaMotte, 2000). The nitrite ion (NO_2^-) content (in mg g^{-1} DW) of each leaf sample was calculated using the formula:

$$NO_3^- \text{ content (in mg } g^{-1} \text{ DW)} = \frac{C \times V}{M}$$

where, C was the concentration of NO_2^- in the sample (in mg g^{-1} DW), V was the total volume of the sample solution (100 ml), and M was the weight of the sample (1.0 g; Radojevic and Bashkin, 1999).

Mineral content measurements

Phosphorus (P) concentrations were measured spectrophotometrically using the ammonium vanadate-molybdate method (Gericke and Kurmies, 1952). Sodium (Na^+) and potassium (K^+) ion concentrations in leaves were estimated using a Perkin-Elmer Model 52-A Flame Photometer (Glenbrook, Stamford, CT, USA; Page *et al*., 1982). Mineral contents were expressed in mg g^{-1} DW leaf tissue.

Anti-oxidant enzyme activities in leaves

For enzyme extraction and assays, 500 mg of each fresh leaf tissue sample was frozen in liquid nitrogen, then ground in 4 ml of 50 mM phosphate buffer (pH 7.0), 1% (w/v) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid. The homogenate was centrifuged at 15,000 × *g* for 30 min, and the supernatant was used for all enzyme assays.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), according to Stewart and Bewley (1980). Each reaction mixture (3 ml) contained 13 mM methionine, 75 mM NBT, 100 mM EDTA, and 50 µl of enzyme extract in 50 mM phosphate buffer (pH 7.8). The reaction was started by adding 2 mM riboflavin and exposing the cuvette to a 15-W fluorescent lamp for 10 min. The absorbance of each reaction mixture was then measured at 560 nm. One Unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of the photochemical reduction of NBT.

Catalase (CAT; EC 1.11.1.6) activity was measured by following the decomposition of H_2O_2 measured over 1 min as the decrease in absorbance at 240 nm, according to Change and Maehly (1955). Each CAT reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2. CAT activity was expressed in Units mg^{-1} FW.

POD activity was assayed in each 3 ml reaction containing 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2, and 0.15 ml of the leaf extract above, according to Polle *et al*. (1994). Peroxidase (POD; EC 1.11.1.7) activity was measured by following the change in absorbance at 470 nm due to oxidation of the guaiacol for 1 min. POD activity was expressed in Units mg^{-1} FW.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) for a randomised complete block design, after testing for homogeneity of error variances according to the procedure outlined by Gomez and Gomez (1984). Significant differences between treatments were compared at $P \leq 0.05$ by Duncan's multiple range test.

RESULTS AND DISCUSSION

Three consecutive exogenous applications of 5.0 mM proline (Pro) to run-off at 20, 30, and 40 DAS enhanced endogenous Pro levels in common bean plants and increased their tolerance to salinity.

A reduction in the DW of common bean seedlings under saline conditions indicated an inhibition of growth. Salinity had adverse effects not only on seedling biomass, but also on other morphological parameters such as shoot length, root length, and plant DW. Moderately saline (EC = 6.03) and strongly saline (EC = 8.97) soils caused significant reductions ($P \leq 0.05$) in the shoot lengths, root lengths, and DWs of common bean seedlings compared to the non-saline treatment (EC = 1.84; Table II). The data confirmed previous results reported by several authors with faba bean (Bekheta *et al*., 2009; Abdelhamid *et al*., 2010) and common bean (Gama *et al*., 2007; Khadri *et al*., 2007; Kaymakanova and Stoeva, 2008). Exogenous spray applications of 5.0 mM Pro significantly improved all growth parameters in medium or high salt-stressed bean plants. The effects of Pro on the growth parameters measured here were more pronounced in 2011 than in 2010, especially under the highest level of salinity (EC 8.97; Table II). It is probable that Pro would have been absorbed by the developing seedlings, where it maintained water status by increasing the influx of water and reducing the efflux of water under salt-induced water-limiting conditions (Chen and Murata, 2008). Furthermore Pro might have protected cell membranes against ion toxicity and salt-induced oxidative stress, increased cellular growth (Banu *et al*., 2009), and thus increased the growth of common bean plantlets.

Chlorophyll plays a vital role in photosynthesis. Chlorophyll contents were significantly reduced by increasing the salt concentration in the nutrient soil compared to bean plants grown in non-saline soil (EC = 1.84 dS m^{-1}; Table II). These results agree with Sadak *et al*. (2010) and Ghassemi-Golezaniet *et al*. (2012)

It was clear that exogenous applications of Pro significantly improved the chlorophyll contents of salt-stressed bean plants, either through stimulating its biosynthesis and/or inhibiting its degradation. This increase could be attributed to the more efficient scavenging of ROS, that would otherwise have destroyed chlorophyll, by anti-oxidant enzymes and cellular anti-oxidants compounds. The effect of Pro on chlorophyll contents may also have been due to stabilising photosynthetic reactions.

Carotenoids assist in the production of vital nutrients related to photosynthesis. These pigments also give certain fruit their red, yellow, or orange colours. Carotenoid contents were significantly reduced by increasing the salt concentration of the soil compared to control plants grown in non-saline soil (EC = 1.84 dS m^{-1}; Table II). These results agree with those of Sadak *et al*. (2010) and Ghassemi-Golezaniet *et al*. (2012). Several reports have indicated that the beneficial effects of additional anti-oxidants on plant survival under salt stress were associated with partial inhibition of the formation of ROS. The application of Pro significantly increased the carotenoid contents of the leaves of bean plants (Table II). It is well-documented that carotenoids are involved in protecting the photosynthetic apparatus against photo-inhibitory damage by singlet oxygen ($^1O_2$), which is produced by the excited triplet state of chlorophyll. Carotenoids directly deactivate singlet oxygen and also quench the excited triplet state of chlorophyll, thus indirectly reducing the formation of singlet oxygen species (Foyer and Harbinson, 1994).

Proline is a compatible osmolyte with an ability to scavenge free-radicals (Matysik *et al*., 2002). The mechanism by which proline reduces free-radical damage includes physical quenching of singlet oxygen ($^1O_2$) and chemical reactions with hydroxyl radicals (Alia *et al*., 2001). Proline has a low ionisation potential and is thus able to form a reversible charge-transfer complex with $^1O_2$ and effectively quench this ROS (Alia *et al*., 2001). Exogenous applications of proline therefore

TABLE II

Effect of three exogenous spray applications of 5.0 mM proline on tap-root length, shoot length, total dry weight, and total leaf chlorophyll and carotenoids contents of leaves of common bean plants grown in three different saline soils in 2010 and in 2011

Treatment		Tap-root length (cm)		Shoot length (cm)		Total dry weight (g $plant^{-1}$)		Chlorophyll (mg g^{-1} FW)		Carotenoids (mg g^{-1} FW)	
Soil EC (dS m^{-1})	Proline (mM)	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
1.84	0	21.1b[†]	22.0a	25.2b	24.9a	8.0b	07.7b	1.19b	1.28b	0.41b	0.43a
	5	29.3a	23.6a	35.9a	26.7a	9.7a	13.7a	1.69a	1.58a	0.58a	0.46a
6.03	0	13.2c	14.7c	16.0d	16.6c	4.2d	05.8c	0.90c	1.04c	0.26c	0.29c
	5	28.4a	17.9b	21.4c	20.3b	6.8c	13.3a	1.60a	1.51a	0.35b	0.35b
8.97	0	04.9e	08.9d	06.0e	10.1e	2.2e	03.2d	0.50d	0.48d	0.10d	0.18d
	5	10.1d	13.0c	12.4d	14.8c	3.4d	05.4c	0.83c	1.06c	0.21c	0.26c

[†]Mean values (n = 6) in the same column for each trait in each year with the same lower-case letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

[#]Measurements were made 50 d after sowing (DAS).

TABLE III

Effect of three exogenous spray applications of 5.0 mM proline on endogenous leaf proline, leaf ascorbic acid (AsA), nitrate ion (NO_3-), and nitrite ion (NO_2-) concentrations in common bean plants grown in three different saline soils in 2010 and in 2011[#]

Treatment		Free proline (mg 100 g^{-1} DW)		AsA (mg 100 g^{-1} FW)		NO_3- (mg g^{-1} DW)		NO_2- (mg g^{-1} DW)	
Soil EC (dS m^{-1})	Proline (mM)	2010	2011	2010	2011	2010	2011	2010	2011
1.84	0	49.8c[†]	52.4c	1.95c	2.03b	1.46d	1.39d	0.081d	0.076d
	5	61.4b	60.7b	2.40b	2.10b	0.78e	0.81e	0.043e	0.044e
6.03	0	58.4b	59.8b	2.58b	2.45a	1.73c	1.64c	0.096c	0.090c
	5	72.3a	74.6a	2.90a	2.65a	1.26d	1.19d	0.070d	0.069d
8.97	0	29.3e	26.9e	0.74e	0.69d	3.11a	2.98a	0.151a	0.163a
	5	41.2d	38.3d	1.61d	1.20c	2.04b	1.97b	0.110b	0.108b

[†]Mean values (n = 6) in the same column for each trait in each year with the same lower-case letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

[#]Measurements were made 50 d after sowing (DAS).

improve crop tolerance to soil salinity and drought by protecting them from the adverse effects of ROS (Szabados and Savouré, 2009).

In addition, proline also affects various physiological processes that result in stress tolerance (Maggio *et al.*, 2002). Proline (Pro) accumulation is an important physiological index for the response of a plant to salt stress (Shi and Yin, 1993), as well as to other types of stress. Salinity markedly increased Pro contents in different salt sensitive and tolerant species or cultivars, with greater accumulation in salt tolerant plants (Ashraf and Harris, 2004; Mansour *et al*., 2005). Our results (Table III) imply that saline stress, or the exogenous application of Pro, increased the accumulation of Pro in the leaves of bean plants. Increased levels of Pro under salt stress were also reported in two wheat cultivars by Khatkar and Kuhad (2000). It was suggested that the accumulation of Pro may be caused by increased proteolysis or by decreased protein synthesis. Higher concentrations of Pro under salt stress are favourable to plants, as Pro participates in the osmotic potential of leaves and thus in osmotic adjustment. In addition to its role as an osmolyte, Pro can also protect enzymes and increase membrane stability under various conditions. Pro may also assist in the non-enzymic detoxification of free-radicals (Durgaprasad *et al*., 1996; Khan *et al*., 2002).

Ascorbic acid is an anti-oxidant which protects plants against oxidative damage resulting from aerobic metabolism, photosynthesis, and a range of pollutants. Increasing soil salinity caused significant reductions in endogenous concentrations of Pro and ascorbic acid (AsA) compared to control plants (Table III). Spraying common bean plants with 5.0 mM Pro significantly increased endogenous levels of both Pro and AsA compared to water-sprayed, non-salinised and salinised bean plants (Table III). The anti-oxidant activity of leaves is an important aspect of their nutritional value since anti-oxidant molecules have a critical role in the detoxification of free-radicals in both plants and humans (Smirnoff, 1995). The AsA pool is a pivotal component of the cellular anti-oxidant defence system. While Maggio *et al.* (2002) reported that little is known about salt-induced variations in the AsA contents of plants, our findings show that AsA concentrations were increased in control or salinised common bean plants following the application of Pro.

Measures to reduce leaf nitrate (NO_3^-) and nitrite (NO_2^-) ion concentrations are important for vegetable production. The effects of NO_3^- ions and their toxic metabolites on human health have been discussed by Santamaria (2006). Higher levels of soil salinity significantly increased leaf NO_3^- and NO_2^- ion concentrations compared to control leaves (EC = 1.84 dS m^{-1};Table III). In the present study, NO_3-N accumulated in bean plants grown under both saline conditions [moderately saline (EC = 6.03) and strongly saline (EC = 8.97)] compared to non-saline control values. This might be due to a mechanism developed by plants to overcome the osmotic stress caused by salinity, while a decrease in NO_3-N might be related to the antagonistic relationship between toxic Cl^- and NO_3^- ions (Meloni *et al*., 2004). Salt stress has been reported to decrease nitrate reductase (NR) activity in leaves (Gouia *et al*., 1994) and, consequently, to increase NO_3^- ion accumulation, as shown here. However, spraying bean plants with 5.0 mM Pro resulted in significant reductions in the concentrations of NO_3^- and NO_2^- ions compared to non-sprayed (Pro-free) control plants (Table III). Foliar applications of Pro improved nitrogen metabolism and decreased NO_3^- ion contents in Japanese pear leaves (Takeuchi *et al*., 2008). Exogenous Pro has also been shown to protect NR activity *in vitro,* which, in turn, would reduce NO_3^- ion concentrations and their assimilation (Sharma *et al*., 1998).

It has been shown that the application of Pro can improve salt tolerance by ameliorating the activites of some anti-oxidant enzymes and protecting photosynthetic activity (Ben Ahmed *et al*., 2010; Kumar *et al*., 2010). In addition, Pro may interact with other stress metabolites and/or their precursors and activate their biosynthetic pathways (Jaleel *et al*., 2009). We have confirmed that Pro acts to increase chlorophyll and carotenoid contents, and anti-oxidant enzyme activities, and to reduced NO_3^- and NO_2- ion levels in bean plants exposed to salt stress.

The exclusion of Na^+ ions, and higher K^+:Na^+ ratios in bean plants grown under saline conditions have been confirmed as important selection criteria for salt tolerance (Abdelhamid *et al.*, 2010). Table IV shows that P and K^+ ion concentrations decreased significantly with increasing soil salinity. In contrast, Na^+ ion levels in leaves increased with increasing salinity. Exogenous applications of Pro significantly increased concentrations of P and K^+, and the K^+:Na^+ ratio, and decreased Na^+ ion levels in salt-affected plants. The results shown in Table IV concur with data on faba bean (Abdelhamid *et al*., 2010) and soybean (Gaballah *et al*., 2011) and indicate that salt tolerance is associated with an enhanced K^+:Na^+ ratio. The ability of a plant to limit the transport of Na^+

TABLE IV

Effect of three exogenous spray applications of 5.0 mM proline on leaf P, K^+, Na^+ concentrations and K^+:Na^+ ratios in the leaves of common bean plants grown in three different saline soils in 2010 and in 2011

Treatment		P (mg g^{-1} DW)		K^+ (mg g^{-1} DW)		Na^+ (mg g^{-1} DW)		K:Na ratio	
Soil EC (dS m^{-1})	Proline (mM)	2010	2011	2010	2011	2010	2011	2010	2011
1.84	0	4.19b[†]	3.98b	6.93a	7.04a	2.24d	1.98d	3.09b	3.56b
	5	6.09a	5.84a	7.05a	7.28a	1.19f	1.12e	5.92a	6.50a
6.03	0	2.15d	2.26d	5.34b	6.01b	2.95c	2.69c	1.81d	2.23d
	5	3.41c	3.19c	5.59b	6.81a	1.94e	1.88d	2.88c	3.62c
8.97	0	1.63e	1.50e	3.14d	4.12d	6.64a	5.09a	0.47f	0.81f
	5	2.24d	2.19d	3.98c	4.73c	3.99b	3.14b	1.00e	1.51e

[†]Mean values (n = 6) in the same column for each trait in each year with the same lower-case letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.
[#]Measurements were made 50 d after sowing (DAS).

into its shoot is important to maintain a high growth rate and to protect metabolic processes from the toxic effects of Na^+ ions (Razmjoo *et al*., 2008). This could be attributed to the ability of roots to exclude Na^+ from the xylem sap flowing to the shoot, which implies better growth of the shoot than the root (Kaya *et al*., 2007). The results here demonstrate that exogenous applications of Pro under non-saline or saline stress conditions resulted in increased P and K^+ levels and higher K^+:Na^+ ratios, but lower concentrations of Na^+ (Table IV). Thus, Pro caused a reduction in Na^+ ion absorption and toxicity. This could explain the mitigating effects of Pro on the growth of bean plants in saline soils. The antagonistic relationship between Na^+ and K^+ ions, as a result of Pro treatment, indicates that Pro could play a role in modifying K^+:Na^+ ratios under salt stress, which is reflected in reduced membrane damage and higher water contents under salinity stress.

Plant cells possess a variety of defence strategies against the oxidative injury caused by salinity stress. Such strategies involve enzymes such as SOD, CAT, and POD which degrade superoxide radicals and H_2O_2, respectively, as well as various anti-oxidants including -tocopherol, AsA, and polyphenolic compounds (Mittler, 2002). Strengthening plant defence mechanisms against oxidative damage, especially when plants are exposed to salinity stress, may be achieved by exogenous applications of anti-oxidants. Senescence-associated parameters can be retarded by anti-oxidants. Moderate salinity stress (EC = 6.03 dS m^{-1}) significantly increased SOD, CAT, and POD activities in the leaves of common bean plants (Table V). However significant decreases in the activities of all anti-oxidant enzymes (SOD, CAT, and POD) were seen at the highest level of soil salinity (EC = 8.97 dS m^{-1}). Three spray applications of exogenous Pro were accompanied by significant increases in SOD, CAT, and POD activities in saline-affected and non-saline-treated control (EC = 1.84 dS m^{-1}) bean plants. The same trends were observed over both seasons (2010 and 2011). The activities of anti-oxidant enzymes, especially SOD, might be inhibited by the rate of $O_2^{\bullet-}$ generation. SOD is the first line of defence against ROS. Changes in SOD activity and/or in the amount of the enzyme have been identified as indicators of oxidative stress (Bowler *et al*., 1992). However, SOD activities increased in both Pro-treated and non-Pro treated salinised plants (Table V). High activities of the enzymes involved in H_2O_2-scavenging have been described in several species. CAT, in concert with peroxidases and other enzymes, destroys the H_2O_2 produced by SOD and other enzymes (Foyer *et al*., 1994). In the present investigation, CAT and POD activities increased (Table V) following applications of Pro under normal or saline conditions. Hence, it is proposed that CAT and POD may play important roles in the rapid defence responses of plant cells against oxidative stress (Zabalza *et al*., 2007). The greater inhibition of anti-oxidant enzymes in water-sprayed control plants under salinity stress compared to Pro-treated plants indicates increased inactivation of the anti-oxidant enzymes by ROS in the former (Djanaguiraman *et al*., 2005). This might be due to the toxic effects of H_2O_2 or other harmful ROS which impair enzyme activities (Noctor and Foyer, 1998). The results of the present study strongly suggest that Pro-sprayed plants have a greater potential to eliminate ROS through higher CAT and POD activities.

CONCLUSIONS

Three exogenous spray applications of 5.0 mM proline at 20, 30, and 40 DAS alleviated oxidative stress and enhanced the growth of common bean plants grown in

TABLE V

Effect of three exogenous spray applications of 5.0 mM proline on the specific activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) in the leaves of common bean plants grown in three different saline soils in 2010 and in 2011

Treatment		SOD (Units mg^{-1} FW)		CAT (Units mg^{-1} FW)		POD (Units mg^{-1} FW)	
Soil EC (dS m^{-1})	Proline (mM)	2010	2011	2010	2011	2010	2011
1.84	0	3.4d[†]	4.0d	6.2c	5.8d	1.8c	2.1cd
	5	6.4b	6.2b	9.8a	10.1b	2.0bc	4.2a
6.03	0	4.8c	5.1c	8.8b	7.9c	2.3b	2.4bc
	5	7.6a	7.4a	10.4a	12.3a	4.5a	2.7b
8.97	0	1.8e	1.3f	4.2d	3.6e	0.8d	1.0e
	5	2.9d	2.7e	6.1c	5.4d	1.8c	1.9d

[†]Mean values (n = 6) in the same column for each activity in each year with the same lower-case letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.
[#]Measurements were made 50 d after sowing (DAS).

soils with high levels of salinity. Proline sprays also increased the activities of three key anti-oxidant enzymes (SOD, CAT, and POD) and the concentrations of carotenoids, chlorophyll, ascorbic acid, and endogenous proline. All of these features enabled the seedlings to reduce the adverse effects of soil salinity. Applications of Pro also increased concentrations of P and K^+, raised the K^+:Na^+ ratio, and decreased Na^+ ion levels in the leaves of salt-treated plants. Based on these findings, we recommend the use of 5.0 mM proline sprays in commercial formulations to enhance plant growth and the production of common bean under salt stress conditions.

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