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Improvement of Salt Tolerance in *Vicia faba* (L.) Plants by Exogenous Application of Polyamines

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SALINITY stress causes physiological drought and disturbances in plant physiology that lead to a reduction in plant growth. To search about an effective method to increase salt tolerance of *Vicia faba* (L.) plants, the effect of soaking the seeds in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on growth, drought tolerance, contents of free proline, total soluble sugars, starch, endogenous Spm and Spd, and antioxidant system in plants grown under salt stress of a saline calcareous soil was studied. Spm or Spd alleviated the adverse effects of salt stress to convergent degrees. Soaking faba bean seeds in either Spd or Spm increased all plant growth characteristics and the activities of antioxidant enzymes compared to the control (seeds soaked in distilled water). In addition, soaking seeds in either polyamine significantly increased membrane stability index, relative water content, contents of ascorbic acid, glutathione, endogenous Spm and Spd. All these improved parameters reflected in enhanced yield and its components. In contrast, electrolyte leakage, concentrations of protein, starch, malondialdehyde and hydrogen peroxide were reduced compared to the control. Data of the present study also show that, the variety Giza 429 exhibited better results than the variety Giza 40, concluding that Giza 429 was more salt-tolerant compared to Giza 40. These results are important as the potential of Spd or Spm to mitigate the deleterious effects of soil salinity stress offer an opportunity to increase the faba bean tolerance to growth under saline conditions.

Keywords: Antioxidant system, Salinity, Spermidine, Spermine, Faba bean varieties.

Salinity stress is one of the most serious abiotic stress factors that limit crop productivity by reduction in plant growth and development (Parida & Das, 2005). Salt stress imposes two restrictions on plants; the hyperosmotic impact because of lower soil water potential and the hyperionic impact due to direct toxicity and antagonism of ions, which cause nutrient imbalance (Neumann, 1997). It affects plant physiology via osmotic and ionic stress at both the whole plant and cellular level. It generates a physiological drought (or osmotic stress) by affecting the water relations of the plant (Munns, 2002). The accumulation of toxic salt concentrations in the leaf apoplasm leads to dehydration, loss of turgor, the death of cells and tissues and consequently the whole plant. Photosynthesis is one of the most severely affected processes during salinity stress (Sudhir & Murthy, 2004) through the decreased level of chlorophyll (Rady, 2011), the

inhibition of various enzymes, including RuBisCO (Soussi *et al.,* 1998), and the closure of stomata, thereby decreasing the leaf intercellular CO_2 pressure (Bethkey & Drew, 1992). These altered processes lead to poor plant growth and productivity. However, lipid peroxidation and the antioxidant system of plants (*e.g.,* antioxidant enzymes and non-enzymatic low molecular weight antioxidants) have been reported to be stimulated by salt stress (Sairam *et al.,* 2005; Rady, 2011 and Rady *et al.,* 2013).

Polyamines (PAs) such as spermine (Spm) and spermidine (Spd) are plant growth regulators, low-molecular-weight polycations ubiquities in all living organisms (Galston *et al.,* 1997). The relationships between PAs and environmental stresses have been studied (Pandolfi *et al.,* 2010 and An *et al.,* 2012). Biosynthesis of PAs has been reported as an integral part of plant's response to salt stress (Alca´zar *et al.,* 2010). The increases in biosynthesis of PAs might protect the plants from salt stress damages by scavenging the harmful free radicals, maintaining the membrane stability and cellular structures, keeping the balance of cation-anion (Bouchereau *et al.,* 1999), regulating the ion channels and inducing the ATP synthesis (Lopatin *et al.,* 1994). Few studies on the exogenous application of free PAs have been focused on alleviating the negative effects of salt stress (Verma & Mishra, 2005; Iqbal *et al.,* 2006; Duan *et al.,* 2008 and Amri *et al.,* 2011), although the contribution of free PAs in osmotic adjustment and the internal changes of these compounds within the plant tissue under the salt stress has been confirmed (Janicka-Russak *et al.,* 2010).

Faba beans (*Vicia faba* L.) are considered popular legume foods consumed worldwide as an important protein source for human and animal nutrition. Seeds of faba beans are rich in carbohydrates, proteins and minerals (Broughton *et al.,* 2003). Saxena *et al.* (1993) reported that there is a considerable variability in salinity tolerance among legumes, however, others considered them either sensitive or only moderately tolerant to salinity (Subbarao & Johansen, 1993). *V. faba* plants are proved to be moderately sensitive legume to salinity (Delgado *et al.,* 1994), exhibiting a reduction in plant growth up to 50% under 6.7 dS m^{-1} salinity (Mass & Hoffman, 1977). Breeding for salt tolerance in crops has usually been limited by the lack of reliable traits for selection (Noble & Rogers, 1992). It is necessary to determine differences in resistance mechanisms between the genotypes and to incorporate characters that improve tolerance into reasonably high-yielding backgrounds. The differences in the salt tolerance of faba bean genotypes were observed by Gaballah & Gomaa (2005).

Nowadays in Egypt, there is a tendency to expand the cultivated area for many crops, including faba beans in newly-reclaimed soils, although most of these soils are affected by salinity, which is considered a global real problem that requires urgent solutions. Despite attempts carried out to identify varieties of faba bean plants through experiments to be grown in the newly-reclaimed saline soils, there is only a limited number of varieties that have been developed with

improved tolerance. Therefore, the present study was designed to examine the influence of PAs (*e.g.*, Spm and Spd), applied by seed soaking, on seedling growth, cell membrane disorders, drought tolerance, osmoprotectant contents and the activities of the antioxidant system in two varieties of faba bean plants grown on a saline calcareous soil (ECe = 8.48 - 8.55).

Materials and Methods

Plant material, growing conditions, experimental design and treatments

Two field experiments were conducted in two successive seasons (2013/2014 and 2014/2015) at the Experimental Farm of Faculty of Agriculture, Fayoum University, Southeast Fayoum (29° 17′N; 30° 53′E), Egypt. The daily temperatures averaged 21.2° ± 2.6 °C and 22.1° ± 2.8 °C, and the daily relative humidity averaged 58.4 ± 5.1% and 60 ± 4.9% in both seasons, respectively. Healthy seeds of two varieties (*i.e.*, Giza 40 and Giza 429) of faba bean (*V. faba* L.) were sown after soaking treatments on 23 and 20 October 2013 and 2014, respectively. Seeds were obtained from Field Crop Research Institute, Agricultural Research Centre, Giza, Egypt. Seeds were selected for uniformity by choosing those of equal size and of the same color and were washed with distilled water, sterilized in 1% (v/v) sodium hypochlorite for approximately 2 min, washed thoroughly again with distilled water, and left to dry at room temperature. Seeds were subjected to soaking treatments in spermine (Spm; 1.5 mM), spermidine (Spd; 1.5 mM) or distilled water (as a control) for 4 h, and then soaked seeds were air-dried again at room temperature overnight. Uniform, air-dried faba bean seeds were sown in hills spaced 20-25 cm apart, in rows spaced 70 cm apart in 3.0 m × 3.5 m plots, using an equivalent of 120 kg seed ha^{-1} to generate the recommended planting density. Thinning was done before the first irrigation to remain two plants per hill. During soil preparation and plant growth, the soil was supplemented with the full dose of NPK fertilizer according to the recommendations of the Ministry of Agriculture and Land Reclamation [*i.e.*, 450 kg ha^{-1} calcium superphosphate (15.5% P_2O_5), 250 kg ha^{-1} ammonium sulfate (20.5% N), and 120 kg ha^{-1} potassiumsulfate (48% K_2O)]. 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 about 2800 m^3 ha^{-1} in each growing season. All other recommended agricultural practices were followed as recommended by the Ministry of Agriculture and Land Reclamation.

One experimental site was chosen for each season in the same location and the analysis of soil samples were conducted according to Klute (1986) and Page *et al.* (1982). Data of soil analyses are presented in Table 1. Based on the EC_e values, soil was classed as being strongly saline according to Dahnke & Whitney (1988). The experimental design used was split plot arrangement in randomized complete block, with one level of each of Spm or Spd (1.5 mM for each), with three replicate plots.

TABLE 1. Some of the physical and chemical properties of the selected soil before planting in two seasons.

Properties	2013/2014	2014/2015
Physical:		
Sand	76.8	75.3
Silt	12.5	13.5
Clay	10.7	11.2
Soil texture	Sandy loam	Sandy loam
Chemical:		
pH [at a soil: water (w/v) ratio of 1:2.5]	7.66	7.72
ECe (dS m^{-1}; soil-paste extract)	8.48	8.55
Organic matter (%, w/v)	0.94	0.92
CaCO3 (%, w/v)	6.86	6.79
Total N (%, w/v)	0.074	0.070
Available P (mg kg^{-1} soil)	8.57	8.39
Available K (mg kg^{-1} soil)	190	185
Available Fe (mg kg^{-1} soil)	6.30	5.99
Available Mn (mg kg^{-1} soil)	2.35	2.29
Available Zn (mg kg^{-1} soil)	0.95	0.93
Available Cu (mg kg^{-1} soil)	0.43	0.50

Plant growth and yield measurements

From each experimental plot, fifty-day-old plants (n = 9) were carefully removed and dipped in a bucket of water. Plants were shaken gently to remove all adhering soil particles and the lengths of their shoots were measured using a meter scale. Numbers of leaves $plants^{-1}$ were counted. The shoots of plants were weighed to record their fresh weights. They were then placed in an oven at 70 °C until constant weight and the dry weights were recorded. Using a graph sheet, leaf areas were measured manually where the squares covered by the leaf were counted. At the end of each experiment (4 and 2 April 2014 and 2015, respectively), yield and its components (*i.e.*, number of dry pods $plant^{-1}$, 100-seed weight, dry seed yield $plant^{-1}$ and dry seed yield $hectare^{-1}$) were determined.

Determination of membrane stability index, electrolyte leakage, and relative water content

Membrane stability index (MSI) was estimated as described by Rady (2011) using duplicate 0.2 g samples of leaf blade. Samples were placed in test tubes containing 10 ml of double-distilled water. One sample was heated at 40 °C in a water bath for 30 min and the electrical conductivity of the solution was recorded using a conductivity bridge (EC_1). The second sample was boiled at 100 °C for 10 min, and the conductivity was measured (EC_2). The MSI was calculated using the formula:

$$MSI\ (\%) = [1 - (EC_1/EC_2)] \times 100$$

The total leakage of inorganic ions from leaves was determined using the method of Sullivan & Ross (1979). Twenty leaf discs were placed in a boiling tube containing 10 ml deionized water and the electrical conductivity (EC_1) was

recorded. The contents were then heated to 45 - 55 °C for 30 min each in a water bath and the electrical conductivity (EC_2) was recorded. The sample was boiled at 100 °C for 10 min and the electrical conductivity (EC_3) was recorded. Electrolyte leakage was calculated using the formula:

$$\text{Electrolyte leakage (\%)} = [(EC_2 - EC_1)/EC_3] \times 100$$

Fresh 2 cm-diameter fully-expanded leaf discs, excluding the midrib, were used to determine the relative water content (RWC). The discs were weighed (fresh mass; FM) and immediately floated on double-distilled water in Petri dishes for 24 h, in the dark, to saturate them with water. Any adhering water was blotted dry and the turgid mass (TM) was measured. The dry mass (DM) was recorded after dehydrating the discs at 70 °C for 48 h. The RWC was then calculated using the formula (Hayat *et al.*, 2007):

$$\text{RWC (\%)} = [(FM - DM)/(TM - DM)] \times 100$$

Determination of free proline, total soluble sugars and starch concentrations

Proline concentration in fresh leaves was measured by the rapid colorimetric method of Bates *et al.* (1973). Each sample of 0.2 g fresh leaf tissue was extracted 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 added to a test-tube and 2 ml of freshly prepared acid-ninhydrin solution was then added. Each tube was incubated in a water bath at 90 °C for 30 min. The reaction was terminated in an ice-bath. Each reaction mixture was extracted with 5 ml of toluene and vortex mixed for 15s. The tubes were allowed to stand for ≥ 20 min in dark at room temperature to allow separation of the toluene and aqueous phases. Each toluene phase was then collected carefully into a test tube and the absorbance of the toluene fraction was read at 520 nm. The proline concentration in each sample was determined using a standard curve of analytical-grade proline.

Total soluble sugars were extracted and determined according to Irigoyen *et al.* (1992). A 0.2 g sample of fresh leaves was homogenized in 10 ml of 96% (v/v) ethanol and washed with 5 ml 70% (v/v) ethanol. The extract was centrifuged at 3,500 × *g* for 10 min and the supernatant was stored at 4 °C for measurement. Total soluble sugars concentrations were determined by reacting 0.1 ml of the ethanolic extract with 3 ml of freshly prepared anthrone reagent [150 mg anthrone plus 100 ml of 72% (v/v) sulphuric acid] and placed in a boiling water bath for 10 min. After cooling, the absorbance of the mixture was recorded at 625 nm using a Bausch and Lomb-2000 Spectronic Spectrophotometer.

The extraction and measuring of starch concentration, using fresh leaf sample (0.1 g) that was homogenized in 80% ethanol, was conducted according to the method of Hedge & Hofreiter (1962). The absorbance was measured at 630 nm. Glucose was used as a standard solution.

Determination of the extent of lipid peroxidation, and the concentrations of ascorbic acid (AsA), glutathione (GSH), hydrogen peroxide (H_2O_2), spermine (Spm), spermidine (Spd) and shoot sodium (Na^+) concentration

Lipid peroxidation was measured in terms of malondialdehyde (MDA) concentration, a product of lipid peroxidation according to Hodges *et al.* (1999). Each 0.5 g sample of fresh leaf tissue was homogenized in a mortar with 10 ml of 80% (v/v) ethanol. The homogenate was then centrifuged at 3,000 × *g* for 10 min at 4 °C., and the pellet was extracted twice with the same solvent. The supernatants were pooled and 1.0 ml was added to a test-tube containing an equal volume of 20% (w/v) trichloroacetic acid (TCA), 0.01% (v/v) butylated hydroxyl-toluene, and 0.65% (v/v) thiobarbituric acid. All samples were heated at 95 °C for 25 min and cooled to room temperature. The absorbance of each sample was recorded at 440, 532, and 600 nm. The formula given by Hodges *et al.* (1999) was used to calculate the level of lipid peroxidation in nmol MDA ml^{-1}.

The AsA concentration in faba bean leaves was determined using the method of Mukherjee & Choudhuri (1983). Each 0.5 g fresh leaf sample was extracted with 10 ml of 6% (w/v) TCA. The extract was mixed with 2 ml of 2% (w/v) dinitrophenylhydrazine (in acidic medium) followed by the addition of one drop of 10% (w/v) thiourea in 70% (v/v) ethanol. The mixture was then boiled for 15 min in a water bath and, after cooling at room temperature, 5 ml of 80% (v/v) H_2SO_4 was added at 0 °C. The absorbance was recorded at 530 nm. The concentration of AsA was calculated from a standard curve plotted with known concentrations of AsA.

The GSH concentration was determined using the method described by Griffth (1980). Fresh leaf tissue (50 mg) was homogenized in 2 ml of 2% (v/v) metaphosphoric acid and centrifuged at 17,000 × *g* for 10 min. Aliquots of the supernatant were neutralized by adding 0.6 ml of 10% (w/v) sodium citrate to 0.9 ml of the extract. Each 1.0 ml assay contained 700 µl NADPH (0.3 mM), 100 µl of 6 mM 5,5′-dithiobis-2-nitrobenzoic acid, 100 µl distilled water, and 100 µl of extract was stabilized at 25 °C for 3 - 4 min. Then 10 µl of 50 units ml^{-1} GSH reductase was added and the absorbance was recorded at 412 nm. GSH concentration was calculated from a standard curve.

The H_2O_2 concentration was assessed using frozen leaf samples (Velikova *et al.,* 2000). Samples (1.0 g) were ground in an ice bath with 5 ml 0.1 % (w/v) trichloroacetic acid. The homogenates were centrifuged at 12,000 × *g* for 15 min. Then, 0.5 ml of the supernatant was added to 0.5 ml of potassium phosphate buffer (10 mM, pH 7). The absorbance of the supernatant was measured at 390 nm.

The extraction and analysis of Spm and Spd were conducted according to Kim *et al.* (2002). Fresh leaf pieces were collected and weighed (0.05 g), then homogenized with a glass homogenizer at 4 °C in the presence of 0.4 ml 5% (v/v) $HClO_4$. The homogenates were centrifuged at 15,000 × *g* for 20 min at 4°C, then supernatants collected and dansylated. The supernatants were mixed with 0.5% (w/v) dansyl chloride (DsCl) saturated with Na_2CO_3 in a ratio of

1:2:1 (v/v/v) and incubated in the dark at 25 °C for 18 h. L-proline (100 mg ml^{-1}) was then added to the mixtures in a ratio of 1:8 (v/v) and incubated for 30 min. To extract Spm and Spd, benzene was added and the mixture was stirred vigorously for 30 s. The extracted solutions of Spm and Spd were then subjected to thin-layer chromatography (TLC). Uniform amounts of Spm and Spd extracts (50 ml for each) and their standard solutions were applied at several points on preheated silica gel plates. The TLC plates were developed in a chamber containing chloroform and trimethylamine in a ratio of 100:9 (v/v). The Spm–like and Spd-like compounds were identified by the same Rf to that of Spm and Spd standards. After confirmation of the location of Spm and Spd with a UV light source, Spm and Spd containing silica spots were collected and eluted with 4 ml ethyl acetate. The fluorescence of these solutions was measured with a UV-fluorescence spectrophotometer (Model F-3010, Hitachi Co., Tokyo, Japan) at 359 nm exciting and 495 nm emission wavelengths. To prepare the standard curves, Spm and Spd at different weights were dissolved in 5% (v/v) $HClO_4$. The Spm and Spd standards were dansylated, extracted and detected as described above.

For Na^+ ion determination, shoot samples faba bean plants were extracted in 0.1 N nitric acid. Na^+ contents were determined by flame photometry in the samples from green faba bean plants (Taleisnik & Grunberg, 1994).

Enzyme assays

Samples of frozen leaves (1.0 g) were homogenized in 50 mM sodium phosphate buffer [pH 7.8 for superoxide dismutase (SOD), and pH 7 for catalase (CAT)]. The homogenates were centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatants were used to assess the activity of the enzymes. SOD activity was assessed according to the method of Giannopolitis & Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the rate of p–nitro blue tetrazolium chloride reduction at 560 nm.

Catalase activity was determined according to the method described by Cakmak & Marschner (1992). The reaction mixture in a total volume of 2 ml contained sodium phosphate buffer (25 mM, pH 7) and H_2O_2 (10 mM). The reaction was initiated by the addition of 100 µl enzyme extract and the activity was assessed by determining the initial rate of disappearance of H_2O_2 at 240 nm (ε: 39.4 mM cm^{-1}) for 30 s. Samples of 1.0 g fresh leaves were ground in 5 ml Tris-HCl buffer (0.05 M). The homogenates were centrifuged at 10,000 × *g* for 25 min at 4 °C. The supernatants were used to analyze the protein content (Bradford, 1979) and the activity of peroxidase (POX) and ascorbate peroxidase (APX) enzymes. POX activity was assessed according to Kara & Mishra (1976). The reaction mixture consisted of 2.5 ml Tris-HCl buffer (0.1 M), 2.5 ml H_2O_2 (5 mM), 2.5 ml pyrogallol (10 mM) and 50 µl enzyme extract. H_2O_2 dependent oxidation of pyrogallol was followed by a decrease in the absorbance at 425 nm (ε: 12 mM cm^{-1}). APX activity was assessed according to Nakano & Asada

(1981). The reaction mixture consisted of 2 ml sodium phosphate buffer (0.05 M), 0.2 ml H_2O_2 (3%), 0.2 ml ascorbate (0.05 mM) and 0.1 ml enzyme extract. H_2O_2 dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε: 2.8 mM cm^{-1}), where "ε" is the molar extinction coefficient**.**

Statistical analysis

All data were subjected to analysis of variance (ANOVA) for a split plot arrangement in randomized complete block design, after testing for homogeneity of error variances according to the procedure outlined by Gomez & Gomez (1984). Combined analysis of data of the two seasons was conducted and significant differences between treatments were compared at $P \leq 0.05$ by Duncan's multiple range test.

Results

Effect of spermidine (Spd) or spermine (Spm) on growth attributes

For varieties, there are significant increases in growth characteristics (*i.e.*, shoot length, leaves number, leaf area, shoot fresh weight and shoot dry weight) of Giza 429 compared to those of Giza 40. Soaking the seeds of both varieties in 1.5 mM spermidine or 1.5 mM spermine significantly increased all tested growth traits of plants compared to control plants that generated from seeds soaked in distilled water (Table 2). The increases in the above growth characteristics were 73.3, 36.6, 62.1, 102.3 and 89.4%, respectively for Giza 40, and were 66.3, 34.4, 66.7, 93.9 and 86.5%, respectively for Giza 429 compared to their controls. There are no significant differences between the results obtained from Spd and Spm applications.

Effect of spermidine (Spd) or spermine (Spm) on membrane stability index (MSI), electrolyte leakage (EL) and relative water content (RWC)

For varieties, there are no significant differences in RWC and EL between both varieties, while Giza 429 was significantly exceeded Giza 40 for MSI. Plants generated from seeds of both varieties which soaked in 1.5 mM spermidine or 1.5 mM spermine exhibited significant increases in RWC and MSI, while showed significant reduction in EL compared to control plants that generated from seeds soaked in distilled water (Table 3). The increases in the RWC and MSI were 23.7 and 14%, respectively for Giza 40, and 20.7 and 17.6 %, respectively for Giza 429, while the decrease in EL was 38.8% for Giza 40 and 31.1% for Giza 429 compared to their controls. There are no significant differences between the results obtained from Spd and Spm applications.

TABLE 2. Effect of seed soaking in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on shoot length (cm), number of leaves per plant, total leaf area (dm^2), fresh weight (FW; g) and dry weight (DW; g) of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		Shoot length	No. of leaves $plant^{-1}$	Leaf area $plant^{-1}$	Shoot FW	Shoot DW
Variety	**Polyamine**					
Giza 40	Control**	32.2 ± 2.5b*	14.2 ± 0.5b	10.3 ± 1.0b	48.6 ± 3.4b	6.6 ± 0.4b
	Spd	55.8 ± 3.3a	19.4 ± 0.7a	16.7 ± 1.5a	98.3 ± 6.2a	12.5 ± 0.7a
	Spm	53.9 ± 3.1a	18.8 ± 0.7a	16.2 ± 1.4a	94.8 ± 5.9a	11.9 ± 0.6a
	Mean	**47.3 ± 3.0B**	**17.5 ± 0.6B**	**14.4 ± 1.3B**	**80.6 ± 5.2B**	**10.3 ± 0.6B**
Giza 429	Control	35.3 ± 2.6b	15.4 ± 0.6b	11.4 ± 1.0b	53.8 ± 3.7b	7.4 ± 0.5b
	Spd	58.7 ± 3.5a	20.7 ± 0.8a	19.0 ± 1.6a	104.3 ± 6.8a	13.8 ± 0.8a
	Spm	58.2 ± 3.4a	20.3 ± 0.8a	18.6 ± 1.6a	99.5 ± 6.3a	13.5 ± 0.8a
	Mean	**50.7 ± 3.2A**	**18.8 ± 0.7A**	**16.3 ± 1.4A**	**85.9 ± 4.6A**	**11.6 ± 0.7A**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

TABLE 3. Effect of seed soaking in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on the membrane stability index (MSI %), electrolyte leakage (EL %) and relative water content (RWC %) of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		MSI (%)	EL (%)	RWC (%)
Variety	**Polyamine**			
Giza 40	Control**	54.2 ± 2.3b*	10.40 ± 0.21a	58.2 ± 3.0b
	Spd	61.8 ± 2.9a	6.37 ± 0.13b	72.0 ± 4.2a
	Spm	60.7 ± 2.7a	6.42 ± 0.14b	70.9 ± 4.0a
	Mean	**58.9 ± 2.6B**	**7.73 ± 0.16A**	**67.0 ± 3.7A**
Giza 429	Control	56.8 ± 2.7b	9.06 ± 0.37a	61. 3 ± 2.1b
	Spd	66.8 ± 3.6a	6.24 ± 0.23b	74.0 ± 3.6a
	Spm	65.5 ± 3.4a	6.38 ± 0.25b	71.9 ± 3.3a
	Mean	**63.0 ± 3.2A**	**7.23 ± 0.28A**	**69.1 ± 3.0A**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

Effect of spermidine (Spd) or spermine (Spm) on protein, free proline, sugars and starch concentrations

For varieties, there are no significant differences in protein and starch between both varieties, but Giza 429 was significantly exceeded Giza 40 for free proline and total soluble sugars. Plants produced from seeds of both varieties which soaked in 1.5 mM spermidine or 1.5 mM spermine showed significant increases in free proline and total soluble sugars, while showed significant decreases in protein and starch compared to control plants that produced from seeds soaked in distilled water (Table 4). The increases in the free proline and

total soluble sugars were 44.8 and 42.4%, respectively for Giza 40, and 39.2 and 40%, respectively for Giza 429, while the decreases in protein and starch were 17.9 and 26.5%, respectively for Giza 40 and 20.8 and 35.5%, respectively for Giza 429 compared to their controls. There are no significant differences between the results obtained from Spd and Spm applications.

TABLE 4. Effect of seed soaking in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on leaf contents of protein (mg g^{-1} FW), free proline (µg g^{-1} FW), total soluble sugar (mg g^{-1} FW) and starch (mg g^{-1} FW) of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		Protein	Free proline	Soluble sugars	Starch
Variety	Polyamine				
Giza 40	Control**	2.57 ± 0.10a*	197.9 ± 5.2b	29.5 ± 1.2b	61.1 ± 3.7a
	Spd	2.11 ± 0.07b	286.5 ± 8.4a	42.0 ± 2.1a	44.9 ± 2.5b
	Spm	2.13 ± 0.07b	278.4 ± 8.6a	38.9 ± 2.0a	45.3 ± 2.8b
	Mean	**2.27 ± 0.08A**	**254.3 ± 7.4B**	**36.1 ± 1.8B**	**50.4 ± 3.0A**
Giza 429	Control	2.69 ± 0.09a	225.9 ± 6.0b	33.0 ± 1.7b	64.3 ± 3.8a
	Spd	2.13 ± 0.06b	314.5 ± 9.1a	46.2 ± 2.4a	41.5 ± 2.9b
	Spm	2.16 ± 0.06b	308.2 ± 8.6a	44.8 ± 2.4a	42.1 ± 3.1b
	Mean	**2.33 ± 0.07A**	**282.9 ± 7.9A**	**41.3 ± 2.2A**	**49.3 ± 3.3A**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

Effect of spermidine (Spd) or spermine (Spm) on malondialdehyde (MDA), ascorbic acid (AsA), glutathione (GSH) and hydrogen peroxide (H_2O_2) concentrations

For varieties, there are significant increases in the AsA and GSH concentrations and significant decreases in the MDA and H_2O_2 concentrations of Giza 429 compared to those of Giza 40. Plants produced from seeds of both varieties which soaked in 1.5 mM spermidine or 1.5 mM spermine showed significant increases in AsA and GSH concentrations, while showed significant decreases in MDA and H_2O_2 concentrations compared to control plants that produced from seeds soaked in distilled water (Tables 5 and 6). The increases in the AsA and GSH concentrations were 11.3 and 28.4%, respectively for Giza 40, and 43.8 and 57.3%, respectively for Giza 429, while the decreases in MDA and H_2O_2 concentrations were 36.5 and 34.8%, respectively for Giza 40 and 41 and 37.6%, respectively for Giza 429 compared to their controls. There are no significant differences between the results obtained from Spd and Spm applications.

TABLE 5. Effect of seed soaking in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on leaf contents of non-enzymatic antioxidants [ascorbic acid (nmol ascorbate g^{-1} FW), total glutathione (GSH; nmol GSH g^{-1} FW), Spm (nmol/g FW) and Spd (nmol/g FW)] of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		Ascorbic acid	Total GSH	Spm	Spd
Variety	**Polyamine**				
Giza 40	Control**	603.5 ± 10.5b*	153.3 ± 5.0b	92.0 ± 3.8c	157.2 ± 6.1c
	Spd	671.7 ± 12.1a	196.8 ± 6.2a	102.0 ± 3.8b	230.1 ± 9.2a
	Spm	669.1 ± 12.2a	201.3 ± 6.4a	112.9 ± 4.9a	191.3 ± 7.8b
	Mean	**648.1 ± 11.6B**	**183.8 ± 5.9B**	**102.3 ± 4.2B**	**192.9 ± 7.7B**
Giza 429	Control	553.5 ± 10.0b	148.4 ± 4.1b	85.5 ± 3.2c	165.5 ± 5.2c
	Spd	795.9 ± 14.2a	233.5 ± 6.8a	118.3 ± 5.3b	259.2 ± 9.6a
	Spm	781.4 ± 13.0a	244.5 ± 6.9a	142.9 ± 6.5a	202.4 ± 7.3b
	Mean	**710.3 ± 12.4A**	**208.8 ± 5.9A**	**115.6 ± 5.2A**	**209.0 ± 7.4A**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

TABLE 6. Effect of seed soaking in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on leaf lipid peroxidation (MDA; in nmol MDA g^{-1} FW) and hydrogen peroxide (H_2O_2; µM mg^{-1} FW) contents, and shoot contents of sodium (Na^+; mg g^{-1}) of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		MDA	H_2O_2	Shoot Na^+
Variety	**Polyamine**			
Giza 40	Control**	70.2 ± 3.6a*	32.5 ± 2.1a	17.2 ± 0.4a
	Spd	44.6 ± 2.1b	21.2 ± 1.3b	9.9 ± 0.2b
	Spm	45.2 ± 2.2b	21.8 ± 1.4b	10.2 ± 0.2b
	Mean	**53.3 ± 2.6A**	**25.2 ± 1.6A**	**12.4 ± 0.3A**
Giza 429	Control**	66.8 ± 3.1a	30.3 ± 2.4a	15.5 ± 0.5a
	Spd	39.4 ± 2.0b	18.9 ± 1.7b	9.2 ± 0.3b
	Spm	40.6 ± 2.3b	19.5 ± 1.8b	9.5 ± 0.3b
	Mean	**48.9 ± 2.5B**	**22.9 ± 2.0B**	**11.4 ± 0.4B**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

Effect of spermidine (Spd) or spermine (Spm) on leaf Spd and Spm and shoot sodium (Na^+) concentrations

For varieties, there are significant increases in endogenous Spd and Spm concentrations and significant decreases in Na^+ concentration in Giza 429 compared to those in Giza 40. Soaking the seeds of both varieties in 1.5 mM Spd or 1.5 mM Spm significantly increased the concentrations of endogenous Spd and Spm. In addition, each polyamine increased itself significantly

compared to other polyamine. In contrast, Spd or Spm application as seed soaking significantly reduced shoot Na^+ concentration compared to the control (Tables 5 and 6). The increases in the endogenous Spd and Spm were 22.7 and 46.4%, respectively for Giza 40 and were 67.1 and 56.6%, respectively for Giza 429 compared to the control. The reduction in Na^+ concentration was 42.4% for Giza 40 and was 40.6% for Giza 429. There are no significant differences between the results obtained from Spd and Spm applications.

Effect of spermidine (Spd) or spermine (Spm) on antioxidant enzyme activities

For varieties, there are significant increases in the activities of all tested enzymes in Giza 429 compared to those of Giza 40. Soaking the seeds of both varieties in 1.5 mM spermidine or 1.5 mM spermine significantly increased the activities of all tested antioxidant enzymes (*i.e.,* superoxide dismutase; SOD, catalase; CAT, peroxidase; POX and ascorbate peroxidase; APX) of plants compared to control plants that generated from seeds soaked in distilled water (Table 7). The increases in the above enzymatic antioxidants were 52.6, 27.3, 78.5 and 79.1%, respectively for Giza 40, and were 45.6, 49, 79 and 69.2%, respectively for Giza 429 compared to their controls. There are no significant differences between the results obtained from Spd and Spm applications.

TABLE 7. Effect of seed soaking in spermidine (Spd; 1.5 mM) or spermine (Spm; 1.5 mM) on the activities of enzymatic antioxidants [superoxide dismutase (SOD; Units g^{-1} FW), catalase (CAT; µkat mg^{-1} protein min^{-1}), peroxidase (POX; Unit mg^{-1} protein min^{-1}), and ascorbate peroxidase (APX; Unit mg^{-1} protein min^{-1})] in leaves of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		SOD	CAT	POX	APX
Variety	**Polyamine**				
Giza 40	Control**	171 ± 6b*	9.9 ± 0.2b	0.93 ± 0.04b	11.5 ± 0.4b
	Spd	261 ± 9a	12.6 ± 0.3a	1.66 ± 0.06a	20.6 ± 0.7a
	Spm	255 ± 8a	12.3 ± 0.3a	1.63 ± 0.06a	20.1 ± 0.7a
	Mean	**229 ± 8B**	**11.6 ± 0.3B**	**1.41 ± 0.05B**	**17.4 ± 0.6B**
Giza 429	Control	195 ± 7b	10.0 ± 0.4b	1.00 ± 0.07b	13.3 ± 0.5b
	Spd	284 ± 10a	14.9 ± 0.5a	1.79 ± 0.09a	22.5 ± 0.8a
	Spm	275 ± 9a	14.1 ± 0.5a	1.73 ± 0.09a	22.1 ± 0.8a
	Mean	**251 ± 9A**	**13.0 ± 0.5A**	**1.51 ± 0.08A**	**19.3 ± 0.7A**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

Effect of spermidine (Spd) or spermine (Spm) on yield and its components

For varieties, there are significant increases in the yield and its components (*i.e.,* number of dry pods $plant^{-1}$, 100-seed weight, dry seed yield $plant^{-1}$ and dry seed yield $hectare^{-1}$) of Giza 429 compared to those of Giza 40. Soaking the seeds of both varieties in 1.5 mM Spd or 1.5 mM Spm significantly increased the yield and its components of plants compared to control plants that generated from seeds soaked in distilled water (Table 8). The increases in the yield and its

components were 62.5, 35.7, 48.7 and 66.3%, respectively for Giza 40, and were 53.9, 47.4, 51.5 and 62.5%, respectively for Giza 429 compared to their controls. There are no significant differences between the results obtained from Spd and Spm applications.

TABLE 8. Effect of seed soaking in spermidine (Spd) or spermine (Spm) on the yield and its components of two varieties of *Vicia faba* (L.) plants grown in reclaimed-saline calcareous soil (combined data over 2013/2014 and 2014/2015 seasons).

Treatments		No. of dry pods plant^{-1}	100-seed weight (g)	Dry seed yield plant^{-1} (g)	Dry seed yield hectare^{-1} (ton)
Variety	Polyamine				
Giza 40	Control**	15.2 ± 0.7b*	55.7 ± 2.5b	39.4 ± 2.1b	1.72 ± 0.13b
	Spd	24.7 ± 1.2a	75.6 ± 3.1a	58.6 ± 3.9a	2.86 ± 0.21a
	Spm	24.3 ± 1.2a	74.1 ± 3.0a	56.9 ± 3.5a	2.79 ± 0.20a
	Mean	**21.4 ± 1.0B**	**68.5 ± 2.9B**	**51.6 ± 3.3B**	**2.46 ± 0.18B**
Giza 429	Control.	17.8 ± 0.9b	57.8 ± 2.6b	43.1 ± 2.4b	1.92 ± 0.14b
	Spd	27.4 ± 1.5a	85.2 ± 3.3a	65.3 ± 4.0a	3.12 ± 0.26a
	Spm	27.2 ± 1.4a	83.4 ± 3.1a	64.6 ± 3.9a	3.07 ± 0.24a
	Mean	**24.1 ± 1.3A**	**75.5 ± 3.0A**	**57.7 ± 3.4A**	**2.70 ± 0.21A**

*Mean values (n = 9) in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.
**Control = seeds soaked in distilled water.

Discussion

Our results showed that growth attributes of faba bean plants reduced under soil salinity. Under soil salinity stress, faba bean plants cannot continue to grow well. So, we chose to focus on the use of the selected suitable level of each polyamine that gives the best improves on recovery saline effect on faba bean plants. Exogenous application of spemidine (Spd) or spermine (Spm) as seed soaking markedly resulted in alleviating the negative effects of salt stress and improving the growth characteristics of faba bean plants (Table 2). These improved growth characteristics have been found to increase the seed yield and its components (Table 8).

The data of the current study indicate that exogenous pretreatment of Spd or Spm was shown to stabilize plant cell membranes, protecting them from damage due to salt stress. In addition, each of polyamines (PAs) maintained cells in a turgid status and reduced ion leakage from cells (Table 3). Borell *et al.* (1997) suggested that these positive findings may be attributed to the increase in the endogenous Spd or Spm (Table 5), which are suggested to participate in sustaining membrane integrity.

In this study, the activities of antioxidant enzymes (*i.e.*, SOD, CAT, POX and APX) significantly increased in faba bean plants when grown under soil salinity stress. This result confirms the finding of Parida & Das (2005) in regard to the

mutual relationship between higher antioxidant activity and salinity tolerance. Exogenous pretreatment (seed soaking) of Spd or Spm caused to increase the activities of POX, APX, SOD and CAT. Each of the PAs can regulate many enzyme activities by bonding with the enzyme protein or participation in the process of phosphorylation of the enzyme protein (Stark *et al.,* 2011). The SOD enzyme is the first defence against superoxide (O_2^-) radicals, found to catalyze the conversion of O_2^- to H_2O_2 that is subsequently reduced to H_2O by POX (Alscher *et al.,* 2002) using several reductants, including AsA and GSH (Apel & Hirt, 2004). Further, CAT also scavenges H_2O_2 by converting it to H_2O and finally O_2.

The reduction in the activity of H_2O_2 was also another result of the pretreatment of Spd or Spm for faba bean plants. The H_2O_2 is one of the major and most stable ROS and its high concentration leads to oxidative stress through the increase in the lipid peroxidation and the reduction in membrane stability index (Upchurch, 2008 and Dionisio-Sesc & Tobita, 1998).

Each of PAs (Spd or Spm) was observed to negate oxidative injury in plants by acting as direct free radical scavengers (Bors *et al.,* 1989). Therefore, the increased activities of SOD, CAT, POX and APX against ROS seem to be one of the mechanisms in which seed soaking in Spd or Spm found to alleviate salt stress in faba bean plants. The results of this study showed a more important role, to some extent, of Spd than Spm in alleviating salt stress in faba bean plants. This might be attributed to the increased endogenous concentrations of Spd than Spm in faba bean plants (Table 5).

However, many contrasting reports have suggested the change in PAs under salt stress conditions to be a protective mechanism (Kasukabe *et al.,* 2004 and Iqbal *et al.,* 2006). In the current study, salt stress caused a reduction in the concentration of protein in faba bean plants which confirms the finding of Sharma & Dubey (2010), and pretreatment of Spd or Spm increased protein concentration. Furthermore, production of free radicals such as H_2O_2 under salt stress conjugates to proteins, and consequently destruction in their structures (Peltzer *et al.,* 2002). Therefore, the positive influence of Spd or Spm might be due to either preventing the production of free radicals or free radicals scavenging mechanisms, thereafter protecting protein levels.

Under salt stress, soluble sugars tend to increase while starch concentration decreases (Chaves *et al.,* 2009), which is in agreement with our findings. Photosynthesis is the most important phenomenon inhibited under the stress resulting in reducing the whole produced starch and soluble sugars within the seedlings (Demetriou *et al.,* 2007). The modulation of Spd or Spm in reducing the concentration of soluble sugars and increasing the concentration of starch in faba bean plants under salt stress might be due to the constructive roles of Spd and Spm in improving and maintaining the structure and function of photosynthetic system during salinity stress.

Our results showed also that under soil salinity stress, the proline concentration in faba bean plants was increased. In this concern, Zhu (2001) reported that plants accumulate compatible osmolytes such as proline when they are subjected to salinity stress. In addition, proline probably detoxifies plants by scavenging ROS or prevents them from damaging cellular structures. There is strong evidence that proline as an amino acid play an adaptive role in mediating osmotic adjustment and protecting the subcellular structures in stressed plants (Ashraf & Harris, 2004). In our study, Spd or Spm caused to increase the content of proline in faba bean plants showing a mechanism with other mechanisms by which Spd or Spm reduces the negative impact of soil salinity stress, increasing the faba bean yields.

Data of the present study also show that, the variety Giza 429 exhibited better growth characteristics, water relations, antioxidants (enzymatics and non-enzymatics), polyamines, pod and seed yields than the variety Giza 40, concluding that Giza 429 was more salt–tolerant compared to Giza 40.

More work is necessary to find exactly the protecting roles of these PAs (*i.e.*, Spd and Spm) on photosynthetic systems under salt stress, to provide potential new mechanisms of a plant's tolerance to salinity stress, and to define the physiological roles of Spd and Spm in relation to salinity stress.

Conclusion

Among the PAs, Spd or Spm are beneficial to cope with salt stress, but additional studies are needed to separate the best effectiveness of these two PAs as well as their concentrations. Results of this study demonstrate that Spd or Spm reduced the soil salinity stress by increasing the activities of SOD, CAT, POX and APX and reducing the levels of H_2O_2. Indeed, Spd or Spm has shown to effectively protect the faba bean plants against the adverse effects of salt stress, at least partially by protecting proteins and carbohydrates against ROS and positively altering the antioxidant system in plants. Exogenous application of Spd or Spm as a seed soaking solution might be recommended as an effective technique for improving the tolerance of faba bean plants, especially the variety Giza 429 under salinity stress.

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تحسين تحمل نباتات الفول البلدي للملوحة باستخدام البولي أمينات

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يسبب الإجهاد الملحي جفاف فسيولوجي وإضطرابات في فسيولوجيا النبات والتى تؤدى إلى إنخفاض فى نمو النبات. لذلك كان الهدف من إجراء التجربة هو البحث عن الطريقة الفعالة لزيادة تحمل نباتات الفول البلدي للملوحة , تم دراسة تأثير نقع البذور في الإسبرميدين (بتركيز ١,٥ ملليمول) والإسبرمين (بتركيز ١,٥ ملليمول) على النمو ، تحمل الجفاف ، المكونات الكيماوية لكلا من البرولين الحر ، السكريات الذائبة الكلية ، النشا ، الإسبرمين والإسبرميدين الداخلي ومضادات الأكسدة في النباتات النامية تحت إجهاد الملوحة لتربة ملحية جيرية . وقد وجد أن الإسبرمين أو الإسبرميدين قد خفض من التأثيرات المعاكسة للإجهاد الملحى بدرجات متفاوتة . وجد أيضا أن نقع بذور الفول البلدى في الإسبرميدين أوالإسبرمين أدى إلى زيادة كلا من صفات النمو وأنشطة الإنزيمات المضادة للأكسدة بالمقارنة بالكنترول (نقع البذور في الماء المقطر). بالإضافة إلى ما سبق فقد وجد أيضا أن نقع البذور في أي من البولى أمينات المستخدمة زاد معنويا دليل ثبات الغشاء ، محتوى الماء النسبي ، تركيزات كلا من حامض الإسكوربيك الجلوتاثيون ، الإسبرمين و الإسبرميدين الداخلى . مما أدى إلى تحسين المحصول ومكوناتة . بالإضافة إلى أن نقع البذور في أى من البولى أمينات المستخدمة خفض كلا من الإستنزاف الإلكتروليتي ، تركيزات البروتين ، النشا ، الأكسدة الزائدة للدهون ، فوق اكسيد الهيدوجين بالمقارنة بالكنترول. تظهر نتائج هذه الدراسة أن الصنف جيزة ٤٢٩ كان أكثر تحملاً للملوحة مقارنة بالصنف جيزة ٤٠. مما سبق يتضح أهمية إستخدام الإسبرميدين أوالإسبرمين لتخفيف التأثيرات الضارة لإجهاد ملوحة التربة ولزيادة تحمل نباتات الفول البلدى لتلك الظروف المعاكسة.