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Research article

Effective microorganisms enhance the scavenging capacity of the ascorbate–glutathione cycle in common bean (*Phaseolus vulgaris* L.) plants grown in salty soils

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ABSTRACT

No information is available regarding effective microorganisms (EM) influence on the enzymatic and non-enzymatic antioxidant defence system involved in the ascorbate–glutathione cycle under saline conditions. Therefore, as a first approach, this article focuses on the contribution of EM to the scavenging capacity of the ascorbate–glutathione cycle in salt-stressed plants. It investigates some mechanisms underlying alleviation of salt toxicity by EM application. *Phaseolus vulgaris* cv. Nebraska plants were grown under non-saline or saline conditions (2.5 and 5.0 dS m^{-1}) with and without EM application. Lipid peroxidation and H_2O_2 content were significantly increased in response to salinity, while they decreased with EM application in both stressed and non-stressed plants. Activities of ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2) increased under saline conditions; these increases were more significant in salt-stressed plants treated by EM. Activities of monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) decreased in response to salinity; however, they were significantly increased in stressed plants treated with EM. Ascorbate and glutathione contents were increased with the increasing salt concentration; moreover they further increased in stressed plants treated with EM. Ratios of AsA/DHA and GSH/GSSG decreased under saline conditions, whereas they were significantly increased with EM treatment in the presence or in the absence of soil salinization. The EM treatment detoxified the stress generated by salinity and significantly improved plant growth and productivity. Enhancing the H_2O_2-scavenging capacity of the ascorbate–glutathione cycle in EM-treated plants may be an efficient mechanism to attenuate the activation of plant defences.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important crops, which is widely grown worldwide for its greater nutritional value and protein rich seeds. However, it is highly sensitive to soil salinity, which posses a severe threat to its productivity (Maas and Hoffman, 1977). Salinity is considered to be a significant factor affecting crop production and agricultural sustainability in many regions of the world and it reduces the value and productivity of the affected lands (Munns, 2002). Its damaging effects are due to its ionic and osmotic stresses, which severely depress various physiological and biochemical processes such as photosynthesis, protein synthesis and energy and lipid metabolism (Parida and Das, 2005). Salinity stress can also cause multifarious adverse effects on plant metabolism. Of these effects, the production of reactive oxygen species (ROS) is a common phenomenon, which causes oxidative stress (Ashraf, 2009). To cope with ROS and maintain redox homeostasis, plants have developed a well-integrated antioxidant defence system. The ascorbate–glutathione cycle is the key mechanism of scavenging ROS in plant tissues, and its high efficiency is responsible for the alleviation of oxidative damage under abiotic stress (Asada, 2006).

Ascorbate (AsA) and glutathione (GSH) are potent non-enzymatic antioxidant within cell. AsA scavenge most dangerous forms of ROS i.e. •OH, O_2^-, H_2O_2 through the action of APX, while glutathione participate in maintaining cellular AsA pool in reduced state through Asada–Halliwell pathway as well as serve as major thiol disulfide redox buffer in plants (Nocter and Foyer, 1998; Ashraf, 2009). Enhancing the levels of AsA and GSH are correlated with plant adaptation to salinity stress (Talaat and Shawky,

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2013a,b). Moreover, high redox status of the antioxidants ascorbate (AsA/DHA) and glutathione (GSH/GSSG) contributes to prevent oxidative injury and associate with plant salt tolerance (Hernández et al., 2010). At present, the relationship between salt tolerance and an efficient antioxidant system has been shown by D'Souza and Devaraj (2010), Hernández et al. (2010) and Talaat and Shawky (2013a,b); whereas the behavior of the antioxidant system in salt-affected plants treated by EM has received no attention. The present study on antioxidative response of salt-stressed plants treated with EM will be a new and first report.

Effective microorganisms (EM) technology improves crop quality and has great potential to improve chemical and physical properties of the soil. It can improve decomposition of organic amendments and release of plant available nutrients, increase nutrient availability in the rhizosphere of plants, enhance seed germination, emergence and seedling growth, improve biocontrol of plant diseases and pathogens through antagonism and antibiosis, increase plant growth from microbially sythesized hormones (e.g., auxins) and growth factors, detoxify residual phytotoxic substances, and increase production of antioxidants that suppress the adverse effects of free radicals in plant metabolism (Higa, 2004; Javaid, 2006; Hu and Qi, 2013).

However, there is no information concerning the effect of EM application on the behavior of the ascorbate–glutathione cycle in the presence or in the absence of salinity. The main aim of this study was to characterize salt resistance strategies in stressed plants treated with EM by evaluating the enzymatic and non-enzymatic antioxidant defence system involved in the ascorbate–glutathione cycle. It was further directed to establish a possible interrelationship between EM application and oxidative stress tolerance in plants exposed to saline conditions. The present investigation hypothesizes that EM application may protect salt-stressed plants against the oxidative damage induced by salinity, and may improve plant growth and its productivity by enhancing the scavenging capacity of the ascorbate–glutathione cycle in plant cells. This approach will give novel information about plant growth stimulation by EM application under saline conditions.

2. Materials and methods

2.1. Experimental design, plant material and growth conditions

Pot experiment was carried out in the greenhouse of the Department of Plant Physiology, Faculty of Agriculture, Cairo University at Giza, Egypt, during the two successive seasons of 2012 and 2013. The experiment included six treatments (three salinity levels × two effective microorganisms' treatments). Treatments were replicated four times and arranged in a complete randomized design.

Seeds of common bean (*Phaseolus vulgaris* L.) cv. Nebraska were obtained from the Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture, Giza and were sown under three levels of soil salinity [0.1 dS m^{-1} (non-saline), 2.5 and 5.0 dS m^{-1}]. Saline conditions were obtained by adding to the soil a mixture of NaCl, $CaCl_2$ and $MgSO_4$ at the molar ratio of 2:2:1, respectively.

At each salinity level, seedlings (10 days old) were subjected to two effective microorganisms' treatments: treated with EM and untreated plants. To the EM-treated plants (EM+), EM was applied in a formulation designated as EM1, which contained a mixed of benefic microorganisms [photosynthetic bacteria (Rhodopseudomonas spp.), lactic acid bacteria (Lactobacillus spp.), yeast (Saccharomyces spp.), actinomycetes, and fermenting fungi (*Aspergillus* and *Penicillium*)]. It was prepared by the Centralized Management of Afforestation and the Environment, Ministry of Agriculture and Land Reclamation, Giza, Egypt. EM1 stock solution was diluted 1:1000 (EM:water) and was sprayed on plant and soil at the time of irrigation. Untreated plants (EM−) were sprayed with double distilled water.

The soil used was a clay loam (sand 39%, silt 24%, clay 37%), collected from the Faculty of Agriculture, Cairo University Experimental Station, sieved (pore size, 2 mm), diluted with quartz sand (particle diameter < 1 mm; 2:1, soil to sand, v/v), and sterilized by steaming the mixture at 100 °C for 1 h on three consecutive days. Before planting, soil chemical analysis was determined according to Cottenie et al. (1982) and presented in Table 1. Fertilization was carried out by adding ammonium sulfate (20.5% N), calcium superphosphate (15.5% P_2O_5), and potassium sulfate (48% K_2O) at the rate of 1.0, 2.0, and 0.5 g pot^{-1}, respectively, before planting, as well as 1.0 g pot^{-1} ammonium sulfate 30 days after planting. For each pot (containing 10 kg of the soil mixture), four seeds thinned to two after germination were planted on February 24 in both seasons. All pots were irrigated to soil saturation before planting. After planting, irrigation was applied at the appropriate times with tap water to maintain soil moisture near maximum water-holding capacity. The plants were sampled after 40 days of sowing to assess the following parameters.

2.2. Measurement of plant growth parameters

Randomly selected plants per treatment were collected to measure plant height, leaves number $plant^{-1}$ and root length. Total leaf area $plant^{-1}$ was also determined using a leaf area meter (LiCOR 3100; Li-cor, Lincoln, NB, USA). Shoot and root dry weights $plant^{-1}$ were estimated after oven drying at 75 °C for 48 h.

2.3. Plant productivity analysis

At maturity, number of seeds $plant^{-1}$ and seed yield $plant^{-1}$ were recorded.

2.4. Determination of lipid peroxidation and hydrogen peroxide content

Lipid peroxidation rates were estimated by measuring the malondialdehyde (MDA) equivalents according to Hodges et al. (1999). Leaf sample was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3000×*g* for 10 min at 4 °C. The pellet was extracted twice with 80% ethanol. The supernatant was added to a test tube with an equal volume of the solution comprised of 20% trichloroacetic acid, 0.01% butylated hydroxy toluene and 0.65% thiobarbituric acid. Samples were

Table 1
Chemical properties of the used soil under different salinity levels.

Salinity levels EC (dS m^{-1})	pH	$HCO_3^- + CO_3^{2-}$ (mg kg^{-1})	Cl^- (mg kg^{-1})	SO_4^{2-} (mg kg^{-1})	Ca^{2+} (mg kg^{-1})	Mg^{2+} (mg kg^{-1})	Na^+ (mg kg^{-1})	K^+ (mg kg^{-1})	N (mg kg^{-1})	P (mg kg^{-1})
0.1	7.20	204.8	317.1	438.1	93.1	39.1	3.7	28.9	18.7	3.0
2.5	7.40	233.2	699.6	709.9	184.9	93.4	165.1	33.0	15.0	2.7
5.0	7.53	274.1	1009.4	1019.1	409.4	168.7	300.1	38.1	13.5	2.5

heated at 95 °C for 25 min and cooled to room temperature. Absorbance was recorded at 440, 532 and 600 nm. Lipid peroxidation rates equivalent (nmol malondialdehyde ml^{-1}) were calculated by using the formula given by Hodges et al. (1999). The content of H_2O_2 was determined as reported by Jana and Choudhari (1981). Hydrogen peroxide was extracted by homogenizing 0.5 g leaf samples in 3.0 ml of phosphate buffer (50 mM and pH 6.8). The homogenate was centrifuged at 6000×*g* for 25 min. Three milliliters of extract were mixed with 0.1% titanium chloride in 20% (v/v) sulfuric acid and the mixture was again centrifuged at 6000×*g* for 15 min. The absorbance of the solution was read at 410 nm, and compared with that of the calibration curve.

2.5. Assay of ascorbate–glutathione cycle enzymes activity

Leaf tissue (0.5 g) was homogenized in 5 ml of 100 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidine and 1 mM EDTA. The homogenate was centrifuged at 15,000×*g* for 10 min at 5 °C and the supernatant obtained was monitored for the activity of ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2). The activity of ascorbate peroxidase was measured as described by Ramel et al. (2009) in a 3 ml reaction mixture containing 1.5 ml of 0.1 M potassium phosphate buffer (pH 6.8), 0.5 ml of 6 mM ascorbate, 0.5 ml of 12 mM H_2O_2, and 0.5 ml of enzyme extract. Change in absorbance was monitored at 290 nm (ε, 2.8 mM^{-1} cm^{-1}). Monodehydroascorbate reductase activity was assayed according to Hossain et al. (1984) by monitoring the change in absorbance at 340 nm due to NADH oxidation (ε, 6.2 mM^{-1} cm^{-1}) for 4 min in a 1 ml reaction mixture containing 90 mM K-phosphate buffer (pH 7.0), 0.0125% Triton X-100, 0.2 mM NADH, 2.5 mM ascorbate, 0.25 U ascorbate oxidase and enzyme extract. One unit of ascorbate oxidase is defined as the amount that causes the oxidation of 1 μmol of ascorbate to monodehydroascorbate per minute. The activity of dehydroascorbate reductase was determined according to Doulis et al. (1997) by measuring the reduction of dehydroascorbate at 265 nm for 4 min (ε, 14 mM^{-1} cm^{-1}). The reaction mixture consisted of 90 mM K-phosphate buffer (pH 7.0), 1 mM EDTA, 5.0 mM GSH, and enzyme extract. The reaction was initiated by the addition of 0.2 mM dehydroascorbate. Glutathione reductase activity was assayed as described by Foyer and Halliwell (1976). The oxidized glutathione (GSSG)-dependent oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was followed at 340 nm (ε, 6.2 mM^{-1} cm^{-1}) in a 1 ml reaction mixture containing 100 mM sodium phosphate buffer (pH 7.8), 0.5 mM GSSG, 50 μl extract, and 0.1 mM NADPH.

2.6. Estimation of reduced (AsA) and oxidized (DHA) ascorbate contents

Ascorbate (AsA) and dehydroascorbate (DHA) contents were measured using α-α′-bipyridyl-based colorimetric assay (Gillespie and Ainsworth, 2007). Leaf tissue was homogenized under liquid nitrogen and extracted in 6% TCA. After centrifugation at 10,000×*g* for 30 min, 200 μl of supernatant was taken for assay, and after the addition of 100 μl of 75 mM phosphate buffer and 100 μl of 10 mM dithiothreitol (DTT), samples were incubated for 10 min at room temperature to reduce oxidized-ascorbate to ascorbate. Excess DTT was removed by the addition of 100 μl of 0.5% *N*-ethylmaleimide (NEM) whereas 200 μl of water was used in place of DTT and NEM in case of reduced ascorbate contents. Then, 500 μl of 10% TCA, 400 μl of 43% H_3PO_4, 400 μl of 4% α-α′-bipyridyl, and 200 μl of 3% $FeCl_3$ were added to all assay tubes. Blanks were prepared using only 6% TCA and ascorbate standards were made in 6% TCA. All assay tubes were incubated at 37 °C for 1 h and absorbance was read at 525 nm. The concentrations of total ascorbate and reduced ascorbate were calculated from the standard curve of ascorbate, and the concentration of dehydroascorbate was calculated after subtracting values of reduced ascorbate from those of total ascorbate.

2.7. Determination of glutathione (GSH, GSSG and total glutathione) levels

Reduced (GSH), oxidized (GSSG) and total glutathione contents were estimated following the method of Anderson (1985). Leaf tissue (0.5 g) was homogenized in 2 ml of 5% (w/v) sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000×*g* for 10 min and supernatant was collected. To 0.5 ml of supernatant, 0.5 ml of 0.1 M (pH 7.0) reaction buffer, 0.5 mM EDTA and 50 μl of 3 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were added. After 5 min the absorbance was read at 412 nm. To the same tube, 100 μl of NADPH (0.4 mM) and 2 μl GR was added for the determination of total glutathione (GSH + GSSG); the reaction was allowed to run for 20 min. The amount of GSSG was calculated by subtracting GSH from total glutathione concentrations. A standard curve was prepared from varying concentrations of reduced GSH.

2.8. Statistical analysis

All data were statistically analyzed using a two-factorial completely randomized design (Snedecor and Cochran, 1980). Combined analysis was made for the two growing seasons, since the results of the two seasons followed a similar trend. All values are means of four replicates. Significant differences were calculated using the least-significant-difference (LSD) test at $p < 5\%$ level.

3. Results

3.1. The impact of soil salinity and/or EM application on plant growth and productivity

With increasing salinity level, the growth parameters (shoot height, leaves number $plant^{-1}$, total leaf area $plant^{-1}$, dry weight of shoot $plant^{-1}$, root length, and dry weight of root $plant^{-1}$) of *P. vulgaris* plants were reduced gradually; the reductions generated by higher concentration (5.0 dS m^{-1}) of soil salinity were more severe than those generated by the lowest one (Fig. 1 and Table 2). Compared with the control (non-stressed plants), the values of the above mentioned parameters were decreased by 35.6%, 33.3%, 33.6%, 31.0%, 19.4% and 21.3% under 2.5 dS m^{-1} salinity level, respectively, whereas the respective values under 5.0 dS m^{-1} salinity level were 56.7%, 53.3%, 50.8%, 55.2%, 41.1% and 44.3%, respectively. However, EM application to non-stressed or stressed plants significantly ($p < 0.05$) enhanced the values of these parameters, over the respective controls. The toxicity generated by the high soil salinity was almost neutralized by EM treatment. Shoot height was enhanced in EM-treated plants by 33.5%, 85.3% and 127.7%, that of leaves number $plant^{-1}$ by 25.0%, 62.5% and 107.1%, that of total leaf area $plant^{-1}$ by 33.1%, 76.0% and 110.5%, that of shoot dry weight $plant^{-1}$ by 30.2%, 60.8% and 116.0%, that of root length by 16.9%, 31.0% and 64.4%, and that of root dry weight $plant^{-1}$ by 14.8%, 33.3% and 70.6%, at 0.1, 2.5 and 5.0 dS m^{-1} salinity level, respectively, when compared with untreated plants.

Soil salinity significantly reduced number of seeds $plant^{-1}$ and seed yield $plant^{-1}$. However, these attributes were significantly higher in EM-treated than untreated plants in the presence as well as in the absence of saline conditions (Fig. 2). Effective microorganisms' application significantly ($p < 0.05$) increased number of

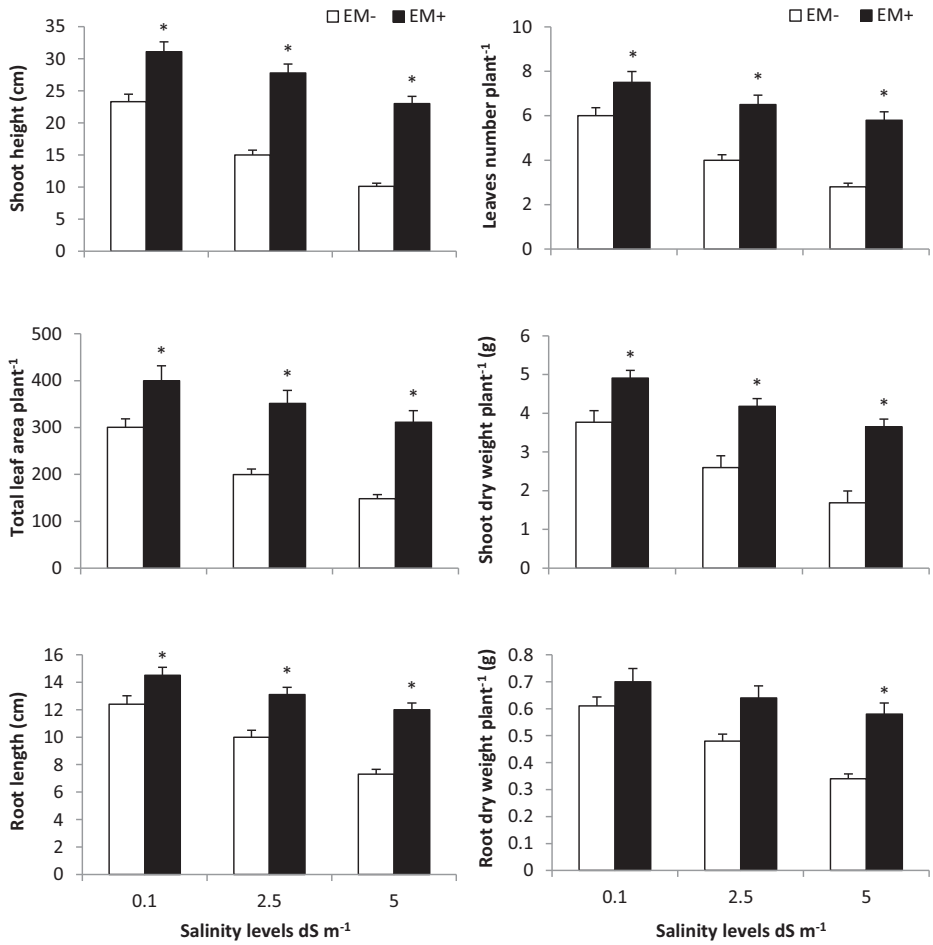

Fig. 1. Changes in the shoot height (cm), leaves number plant^{-1}, total leaf area plant^{-1} (cm^2), dry weight of shoot plant^{-1} (g), root length (cm), and dry weight of root plant^{-1} (g) of commen bean (*Phaseolus vulgaris* L.) cv. Nebraska plants as affected by effective microorganisms' application under different salinity levels. Every column in each graph represents the mean (±SE) of four replicates. Asterisks indicate significant differences at the 0.05 level compared with the untreated plant.

seeds plant^{-1} by 17.4%, 41.2% and 185.7%, and that of seed yield plant^{-1} by 24.8%, 70.4% and 354.5% at 0.1, 2.5 and 5.0 dS m^{-1} salinity level, respectively, when compared with untreated plants.

3.2. Lipid peroxidation and H_2O_2 content in EM-treated plants in the presence and in the absence of soil salinity

Lipid peroxidation and H_2O_2 content were significantly increased in response to salinity, while they were decreased by EM application in both stressed and non-stressed plants (Fig. 3). Soil salinization increased lipid peroxidation and this was significantly ($p < 0.05$) alleviated by EM application. Effective microorganisms' treatment reduced H_2O_2 content by 9.4%, 19.8% and 31.9% and that of MDA by 8.7%, 21.8% and 35.4% compared to values of untreated plants at 0.1, 2.5 and 5.0 dS m^{-1} salinity level, respectively.

3.3. Activities of APX, GR, MDHAR, and DHAR in plants exposed to saline conditions and treated or not with EM

To elucidate the mechanism of redox balance maintenance, the activities of antioxidant enzymes involved in the ascorbate–glutathione cycle were measured. In response to salt treatments, the activities of APX and GR were increased by increasing salinity level. Moreover, their activities in EM-treated plants were significantly ($p < 0.05$) higher than those in untreated ones under saline conditions (Fig. 4). Maximum activities were recorded in EM-treated plants exposed to 5.0 dS m^{-1} salinity level that had 55.6% and 61.1% higher APX and GR activity, over untreated plants, respectively.

The activities of MDHAR and DHAR were decreased with an increase in the concentration of soil salinity. However, when plants were subjected to salt treatments and treated with EM, significant ($p < 0.05$) increases in their activities were observed (Fig. 4). Effective microorganisms' application significantly ($p < 0.05$) enhanced MDHAR activity in EM-treated plants by 100.0% and 187.5% and that of DHAR activity by 70.0% and 260.0% compared to values of untreated plants at 2.5 and 5.0 dS m^{-1} salinity level, respectively.

3.4. Contents of AsA, DHA, GSH and GSSG and ratios of AsA/DHA and GSH/GSSG in EM-treated plants under saline or non-saline conditions

Both salinity stress and EM application increased the contents of GSH and AsA in *P. vulgaris* leaves but these increases were more pronounced in response to EM treatment than in response to soil salinity. Moreover, salt-stressed plants treated with EM had higher GSH and AsA contents in comparison with stressed untreated ones. In the same vein, salinity significantly ($p < 0.05$) increased GSSG and DHA content in leaves of untreated plants. However, their contents in the EM-treated plants were lower than that in untreated ones under all salinity levels. This resulted in significant

Table 2
P-values of the two-way analysis of the plant growth and its productivity characters, hydrogen peroxide content, lipid peroxidation, activities of [ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR)], contents of [reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbate (AsA), and dehydroascorbate (DHA)], and ratios of GSH/GSSG and AsA/DHA parameters measured in commen bean (*Phaseolus vulgaris* L.) cv. Nebraska plants as affected by effective microorganisms' application under different salinity levels. *P*-values are considered significant (<0.05, $n = 4$). 'S': effect of salinity levels; 'EM': effect of effective microorganisms' application; S × EM: effect of the variables' interaction.

	Main-factor effects		Significant interaction
	S	EM	S × EM
Shoot height	<0.0001	<0.0001	0.0018
Leaves number plant^{-1}	0.0005	<0.0001	0.0457
Total leaf area plant^{-1}	<0.0001	<0.0001	0.0082
Shoot dry weight plant^{-1}	<0.0001	<0.0001	0.0338
Root length	<0.0001	<0.0001	0.0338
Root dry weight plant^{-1}	0.0504	0.0145	–
Seeds number plant^{-1}	<0.0001	<0.0001	0.0023
Seed yield plant^{-1}	<0.0001	<0.0001	<0.0001
H_2O_2 content	<0.0001	<0.0001	0.0005
Lipid peroxidation	<0.0001	<0.0001	0.0007
APX activity	<0.0001	<0.0001	0.0002
GR activity	<0.0001	<0.0001	0.0009
MDHAR activity	0.0036	<0.0001	0.0003
DHAR activity	0.0021	<0.0001	0.0001
GSH content	<0.0001	<0.0001	<0.0001
GSSG content	<0.0001	<0.0001	<0.0001
AsA content	<0.0001	<0.0001	<0.0001
DHA content	<0.0001	<0.0001	<0.0001
GSH/GSSG	0.0069	<0.0001	<0.0001
AsA/DHA	0.0262	<0.0001	<0.0001

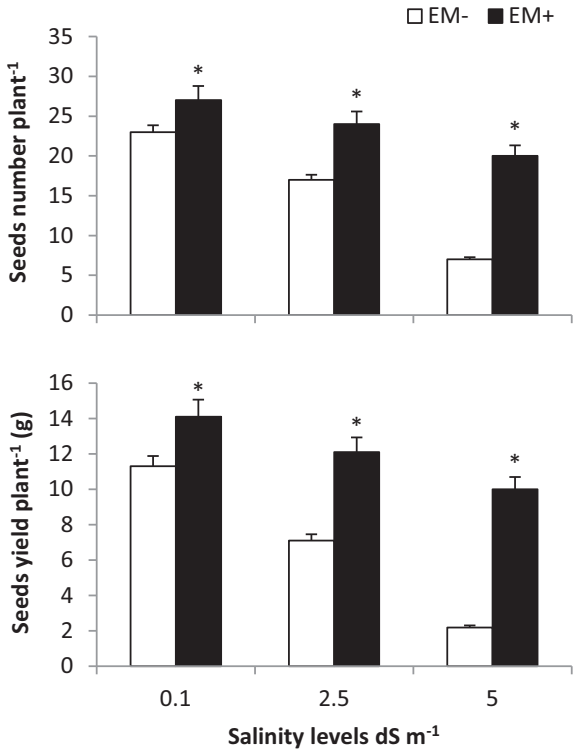

Fig. 2. Changes in the seeds number plant^{-1} and seed yield plant^{-1} (g) of common bean (*P. vulgaris* L.) cv. Nebraska plants as affected by effective microorganisms' application under different salinity levels. Every column in each graph represents the mean (±SE) of four replicates. Asterisks indicate significant differences at the 0.05 level compared with the untreated plant.

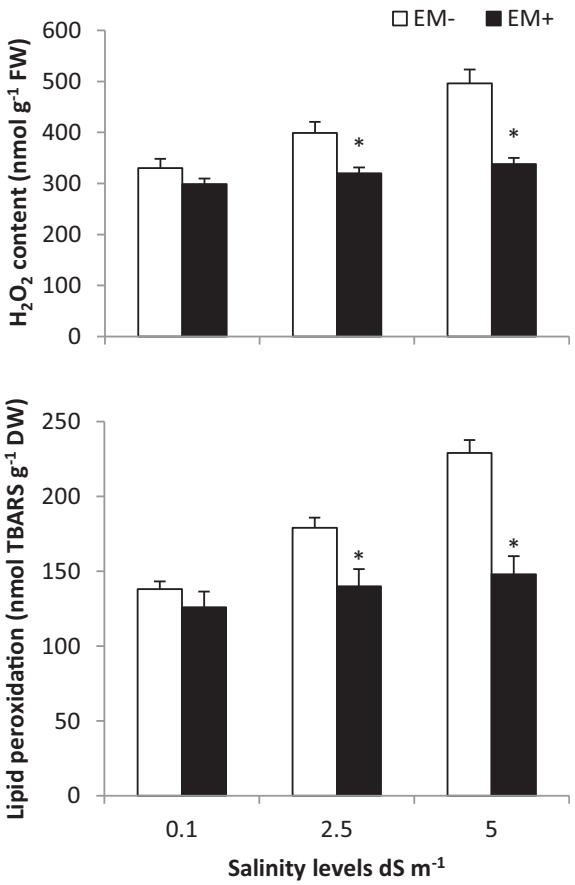

Fig. 3. Changes in the content of H_2O_2 (nmol g^{-1} FW) and lipid peroxidation in leaves of common bean (*P. vulgaris* L.) cv. Nebraska plants as affected by effective microorganisms' application under different salinity levels. Every column in each graph represents the mean (±SE) of four replicates. Asterisks indicate significant differences at the 0.05 level compared with the untreated plant.

($p < 0.05$) increases in the ratios of GSH/GSSG and AsA/DHA in plants submitted to the salt treatments and treated with EM (Fig. 5). The ratio of GSH/GSSG was significantly ($p < 0.05$) enhanced in EM-treated plants by 69.8%, 239.7% and 516.0% and that of AsA/DHA ratio by 66.1%, 191.0% and 502.3% at 0.1, 2.5 and 5.0 dS m^{-1} salinity level, respectively, when compared with untreated plants.

4. Discussion

In recent years, the use of biological methods as a practical way to alleviate soil stresses like salinity has received a greater attention. Moreover, increase in plant salt tolerance is required to sustain the increases in food production in many regions of the world. Common bean (*P. vulgaris* L.) is one of the most important crops and raising its production through increasing the productivity per unit area as well as expanding the cultivated area in newly reclaimed lands may be achieved by using biological strategy. In this investigation, as a first approach, using EM application as a biological and practical way to detoxify the stress generated by salinity may increase the utilization of saline soils and may become an emerging challenge as a promising environmentally friendly method. Overall, this investigation will open new research line aimed at obtaining maximum benefit from the EM under saline conditions.

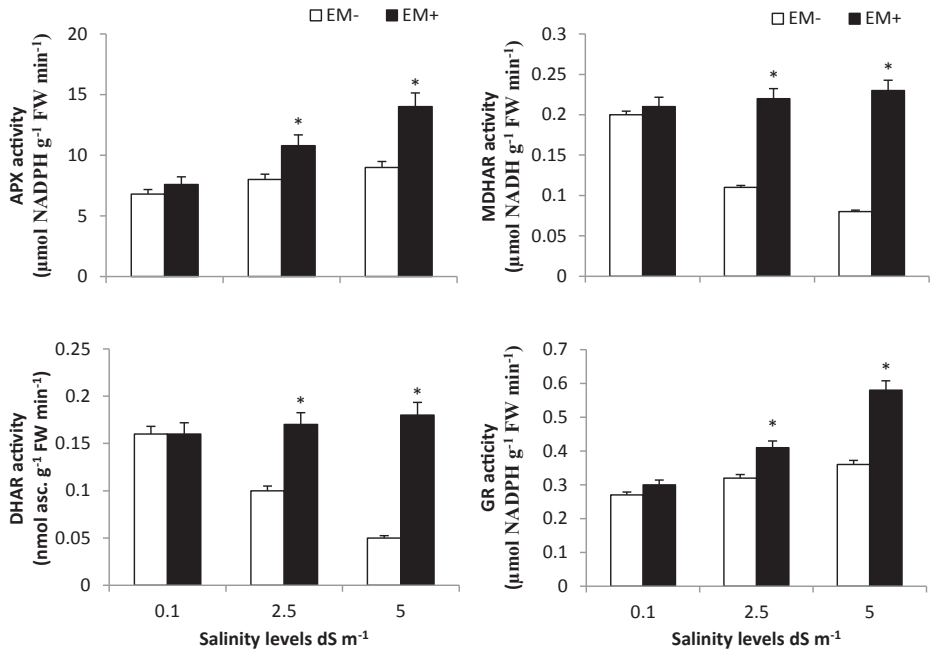

Fig. 4. Changes in the activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in leaves of common bean (*P. vulgaris* L.) cv. Nebraska plants as affected by effective microorganisms' application under different salinity levels. Every column in each graph represents the mean (±SE) of four replicates. Asterisks indicate significant differences at the 0.05 level compared with the untreated plant.

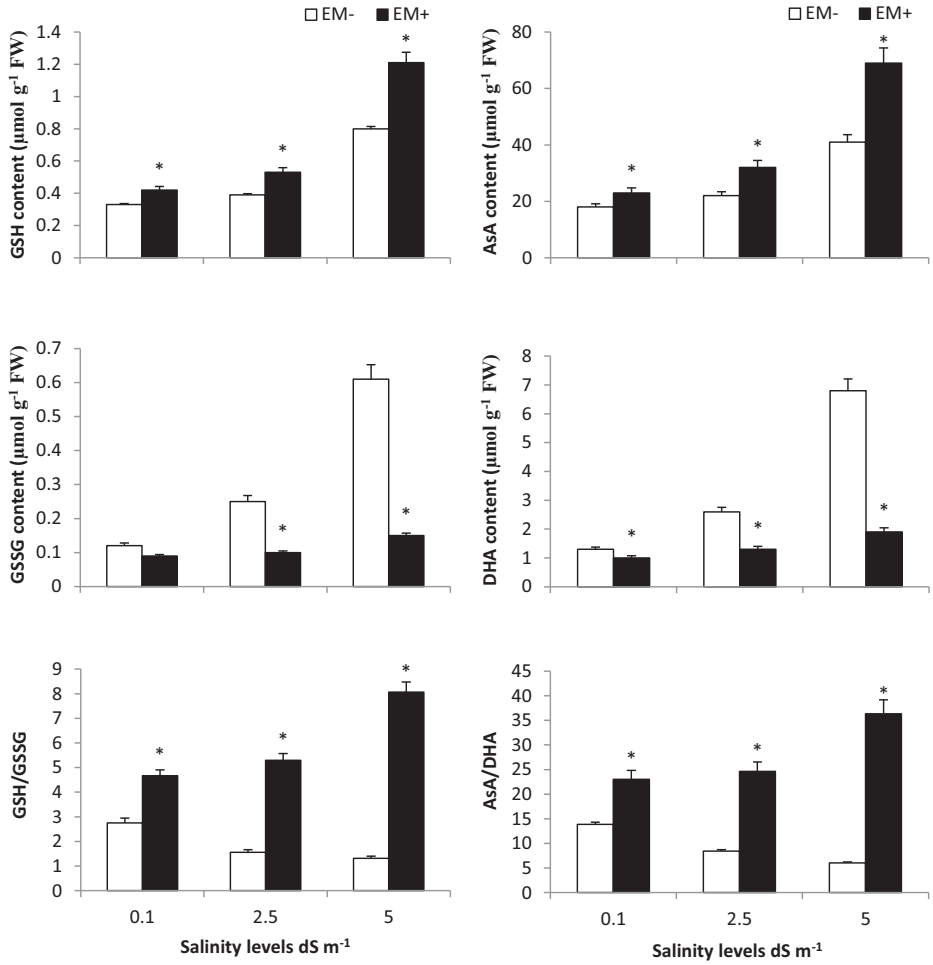

Fig. 5. Changes in the contents of ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) and the ratios of AsA/DHA and GSH/GSSG in leaves of common bean (*Phaseolus vulgaris* L.) cv. Nebraska plants as affected by effective microorganisms' application under different salinity levels. Every column in each graph represents the mean (±SE) of four replicates. Asterisks indicate significant differences at the 0.05 level compared with the untreated plant.

4.1. Salt-induced reduction in plant growth and productivity was reversed by EM application

A major effect of salinity on *Phaseolus vulgaris* plants was the reduction of its growth and productivity. Soil salinization significantly ($p < 0.05$) decreased the plant growth parameters (shoot height, leaves number plant^{-1}, total leaf area plant^{-1}, dry weight of shoot plant^{-1}, root length, and dry weight of root plant^{-1}) as well as its yield attributes (number of seeds plant^{-1} and seed yield plant^{-1}). This result corroborates the findings of (D'Souza and Devaraj, 2010; Hernández et al., 2010; Higbie et al., 2010; Talaat and Shawky, 2013a,b). The resultant damage is generally manifested by different alterations at cellular level including membrane lipid peroxidation (Fig. 3) and over accumulation of ROS such as H_2O_2 (Fig. 3). Indeed, under standard growth conditions, ROS levels in a plant cell are under tight control of scavenging systems that include antioxidative enzymes and antioxidant molecules. However, when ROS are not adequately removed, an effect termed "oxidative stress" may result. Excess ROS formed within cells can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids and DNA. These changes lead to oxidative injuries and result in the reduction of plant growth and development (Mittler et al., 2004).

Additionally, data in Figs. 1 and 2 and Table 2 clearly revealed that EM treatment significantly ($p < 0.05$) increased the plant growth and its productivity and also ameliorated the ill effect caused by soil salinity. Taking into account the well-observed elevation in the activities of APX, DHAR, MDHAR, and GR (Fig. 4) and the improving in the redox state of ascorbate and glutathione (Fig. 5) by EM treatment, this investigation could hypothesized that activation of ROS-scavenging capacity of the ascorbate–glutathione cycle may account for growth stimulation effects of EM under salty soils.

4.2. EM Application detoxified the stress generated by salinity and significantly alleviated the peroxidation of membrane lipids

Because salinity stress increases free radical levels in plants (Asada, 2006; Ashraf, 2009), the damage of membranes was investigated by monitoring lipid peroxidation in leaves. Data in Fig. 3 showed that TBARs content increased as the stress level rose. MDA is an index of lipid peroxidation which has often been used as an indicator of stress-induced damage at the cellular level (Lata et al., 2011). Salt stress is usually accompanied with an increase in lipid peroxidation (Hernández et al., 2010; Talaat and Shawky, 2013a, 2014, Fig. 3).

However, it is interesting to underline that EM-treated plants always exhibited lower H_2O_2 and MDA contents than the corresponding untreated ones, implying lower accumulation of ROS and lower membrane damage in the former than latter plants (Fig. 3). In fact, plants grown in salty soils and treated with EM successfully resist stress as evident from reduced accumulation of MDA. This lower level of lipid peroxidation may suggest that these plants are protected from oxidative damage. Moreover, rise in APX activity (Fig. 4) powered by high AsA content or AsA-redox (Fig. 5) and increasing GR, DHAR, and MDHAR activities (Fig. 4) in these plants could be strong enough to stimulate antioxidant response, and might be instrumental to maintain H_2O_2 at a particular level. Obviously, the absence of salt-induced growth inhibition in EM-treated plants (Fig. 1) might be due to the significantly low levels of membrane lipid peroxidation and H_2O_2 content (Fig. 3).

4.3. EM application modified the activities of APX, GR, MDHAR, and DHAR under saline conditions

To limit oxidative damage under stress conditions, plants have developed a series of detoxification systems that break down the highly toxic ROS (Sairam and Tyagi, 2004). The ascorbate–glutathione cycle system is the key mechanism for scavenging ROS in plant tissues, which includes four important antioxidant enzymes, namely, APX, GR, DHAR, and MDHAR (Asada, 2006). Data in Fig. 4 illustrated that the activities of APX and GR were higher in salt-stressed plants than that in non-stressed ones. This result is generally consistent with those found by Sekmen et al. (2007), Zheng et al. (2009), Hernández et al. (2010) and Talaat and Shawky (2013a,b). On the other hand, the activities of MDHAR and DHAR were decreased markedly due to salinity stress. As the MDHAR and DHAR are equally important in regulating the level of AsA and its redox state under oxidative stress (Asada, 2006; Ashraf, 2009), these decreases in their activities were reflected by decreased AsA/DHA ratio (Fig. 5).

On the other hand, data in the same figure also demonstrated that EM application to plants grown in salty soils significantly ($p < 0.05$) enhanced the activities of APX and GR, which could suggest as an adaptive mechanism to reduce the H_2O_2 content. Moreover, further evidence supporting the involvement of EM treatment in the enhancement of plant tolerance to salinity stress is that the increments in the activities of MDHAR and DHAR. In this concern, Ushimaru et al. (2006) found that overexpression of DHAR increased salt tolerance in *Arabidopsis*. It appears that salt excess inhibited MDHAR and DHAR activities and EM application significantly relieved the salt-induced inhibition on their activities. The increased MDHAR and DHAR activities by EM treatment could suggest that there may be the generation of reduced ascorbate (Fig. 5) which maintained the higher APX activity during salinity stress as APX requires ascorbate as substrate (Asada, 2006; Ashraf, 2009). In EM-treated plants, the increase in APX activity, which could contribute to ascorbate oxidation, was paralleled by an increased capacity for ascorbate regeneration *via* MDHAR, DHAR and GR activities. Hence, the induction of ROS-scavenging enzymes, which involved in the ascorbate–glutathione cycle, could be one of the most important mechanisms for detoxifying ROS synthesized in plants submitted to salt treatments and treated with EM.

4.4. EM application control the contents of AsA, DHA, GSH and GSSG and the ratios of AsA/DHA and GSH/GSSG in plants exposed to soil salinity

Cells and tissues protect themselves against oxidative damage induced by salt stress through up-regulation of a wide variety of antioxidant products (Nocter and Foyer, 1998). Among them, AsA and GSH are essential plant metabolites that regulate major cell functions and play pivotal roles in antioxidant defence (Ashraf, 2009; Talaat and Shawky, 2013a,b). Furthermore, Kocsy et al. (2001) suggested that the change of AsA/DHA and GSH/GSSG is more important in cell resistance to oxidative stress compared with AsA or GSH content. Results presented in Fig. 5 showed that, the decline in AsA/DHA and GSH/GSSG ratios caused by salinity stress was reversed by EM application. Hence, in EM-treated plants, maintenance of elevated levels of AsA and GSH as well as high redox ratios of AsA/DHA and GSH/GSSG could be associated with salt tolerance and contributed to prevent oxidative injury in these plants.

As said previously, higher content of AsA and its redox status (AsA/DHA) were observed in EM-treated plants under saline conditions. It is possible that AsA is directly scavenging H_2O_2 and the AsA redox state is maintained by the increased activities of DHAR and MDHAR (Fig. 4) in EM-treated plants during salt treatments. It should be also noted that, with EM application, neither in the salt-stressed nor in the non-stressed plants did DHA accumulation take place, which could have been due to the induction of DHAR activity in these plants. In this respect, Arisi et al. (1998) reported that the

increase in DHAR activity may be required to sustain cycling of oxidized ascorbate when the flux through the ASC-GSH cycle is increased.

Additionally, data in Fig. 5 implied that stressed plants treated with EM had higher reductive status of GSH (GSH/GSSG), which could be restored by means of higher GR activity (Fig. 4) and increased GSH synthesis (Fig. 5). In this regard, Nocter and Foyer (1998) reported that GR is a key enzyme of the ascorbate–glutathione cycle that protects cells against oxidative damage by maintaining a high GSH/GSSG ratio. Moreover, in salty soils, the GSH concentration was found to be higher in plants treated with EM than that in untreated ones, whereas, GSSG concentration was found to be higher in untreated plants compared to that in treated ones, which indicates that EM-treated plants has efficient antioxidative characteristics which could provide better protection against oxidative damage under saline conditions.

Taken as a whole the data presented in this investigation may illustrate that the activities of MDHAR and DHAR were increased in EM-treated plants under saline conditions (Fig. 4) in order to mitigate oxidative stress and thus regenerating the potent antioxidant AsA (Fig. 5) from its oxidized form (DHA) using GSH as an electron donor; the GSSG thus produced is used by GR (Asada, 2006; Ashraf, 2009). A higher GR activity in these plants (Fig. 4) further demonstrated its superior tolerance mechanism in maintaining redox state. The increased GSH content (Fig. 5) is in turn used for dissipation of H_2O_2 in Halliwell–Asada cycle (Mittler et al., 2004; Asada, 2006) as is indicated by higher APX activity (Fig. 4) in these plants. Hence, the ascorbate–glutathione cycle could be an important antioxidant protection system against H_2O_2 generated in plants submitted to salt treatments and treated with EM. Clearly, the present study indicates that stressed plants treated with EM did not accumulate high levels of H_2O_2 and MDA (Fig. 3) as long as they maintain high AsA/DHA and GSH/GSSG ratios (Fig. 5) *via* enhancing the activities of APX, DHAR, MDHAR, and GR (Fig. 4).

5. Conclusion

In conclusion, this article demonstrates that the salt-tolerant of EM-treated plants may be attributed to its stronger ability to counter lipid peroxidation by its ability to combat oxidative stress *via* enhancing the enzymatic activities of antioxidative enzymes involved in the ascorbate–glutathione cycle and a higher redox status of the antioxidants AsA and GSH. Obviously, EM application could protect the plant cell against the oxidative damage and could improve the survival of plants under harsh environmental conditions. EM application is believed to be the key to sustained environmental improvement and offers a real opportunity for eco-innovation. The present study gives novel information about plant growth stimulation by EM under saline soils and also opens new research lines aimed at obtaining maximum benefit from the EM under salinity or other osmotic stress conditions.

Author contribution

Prof. Neveen B. Talaat: designed the experiment, ran the experiment, determined all the physiological and chemical analysis, analyzed data, and wrote the manuscript.

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