Research Journal of Agriculture and Biological Sciences 1(3): 216-221, 2005

Physiological Responses of Soybean - Inoculation of *Bradyrhizobium japonicum* with PGPR in Saline Soil Conditions

[1]H.S. Han and [2,3]K.D. Lee

[1]Department of Biology, Sunchon National University, Suncheon, Jeonnam 540-742, Korea.
[2]Institute of Agriculture and Life Sciences, Gyeongsang National University, Chinju, 660-701, Korea.
[3]Department of Plant Science, McGill University, Macdonald Campus, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada .

Abstract: Soil salinity is one of the most severe factors limiting nodulation, yield and physiological response in soybean. The possible role of plant growth promoting rhizobacteria (PGPR) in restricting mineral nutrients and thus alleviating soil salinity stress during plant growth has not yet been established. In this study, the beneficial effects of inoculation with salt-stressed PGPR strains were investigated under greenhouse conditions. Salt-stressed plants had significantly decreased plant growth, photosynthesis and mineral uptake with increasing salinity compared to those from non-saline soil. The un-inoculated plants, compared to the inoculated plants, under soil salinity conditions had an increased antioxidant activity and concentration of proline, MDA, GR and APX. The results suggested that inoculation of salt-stressed plants with PGPR strains could alleviate salinity stress.

Key words: Soybean, *Bacillus* sp., *Serratia* sp., salinity, mineral uptake, antioxidant

INTRODUCTION

Soil salinity has been reported to reduce yields, nodulation and the total nitrogen content in legume plants[1,2]. Soybean nodulation has been well known to be extremely sensitive to NaCl. A reduction in inoculation of 50% compared to maximum nodule number and nodule dry weight in soybean occurred with 26.6 mM NaCl in solution culture[1]. Salinity stress also decreases photosynthetic capacity, due to the osmotic stress and partial closure of stomata[3]. Plants can also suffer from membrane destabilization and a general nutrient imbalance[4,5]. Modulation of antioxidant enzyme activity and concentrations are frequently used as indicators of oxidative stress in plants[6]. To protect against oxidative stress, plant cells produce both antioxidant enzymes and non-enzymatic antioxidants[6,7]. Ascorbate peroxidase (APX) is part of the scavenging cycle and catalyzes the reaction of ascorbic acid with H_2O_2, while glutathione reductase (GR) catalyzes the regeneration of ascorbic acid[8].

Plant growth promoting rhizobacteria (PGPR) may improve plant growth and yield by direct and indirect mechanisms[9]. Indirect mechanisms have been observed with most PGPR strains. Direct mechanisms may act on the plant itself and affect growth[10] by means of plant growth regulators, solubilization of mineral materials (PSB and KSB) and fixation of atmospheric nitrogen. These PGPR can also prevent the deleterious effects of one or more phytopathogenic organisms and stressors from the environment[10]. *Bacillus subtilis* can induce plant resistance to stress and produces various plant hormones for growth improvement. Woitke[11] found that a high salinity treatment with *B. subtilis* had even a lower yield despite improved vegetative plant growth. Similar results were reported by Saleh[12] in artichoke and Woitke[11] in tomato to alleviate the toxic effect of salinity. *Serratia proteamaculans* can enhance soybean nodulation and growth under a low temperature stress[13]. These PGPR strains can produce bacterial exopolysaccharides (EPSs) to bind cations including Na^+[14] and it may be envisaged that increasing the population density of EPS-producing bacteria in the root zone would decrease the content of Na^+ available for plant uptake, thus help alleviating salt stress in plants growing in saline environments[15].

Little is known about the inoculation of *Bacillus* sp. and *Serratia* sp. and the PGPR effect on the antioxidant

Corresponding Author: Kyung Dong Lee, Department of Plant Science, McGill University, Macdonald Campus, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, QC H9X 3V9 Canada .
Tel: 514-398-7851 (ext.) 8733 E-mail: Leekd1@hotmail.com

status and physiological response of soybean under soil salinity. The present study was carried out to elucidate the role of PGPR, EPS-producing bacteria in alleviating salinity stress in soybean seedlings grown under soil saline conditions in the greenhouse.

MATERIALS AND METHODS

Soil and bacterial materials: The soil used was Typic Endoaquepts (USDA, Inceptisols). The soil characteristics were pH (1:5 water) 6.5, EC 1.50 dS m^{-1}, organic matter 15 g kg^{-1}, total nitrogen 1.6 g kg^{-1}, CEC: Ca 4.9, K 1.5 and Na 0.4 $cmol^{+}$ kg^{-1}. A basal fertilizer N-P_2O_5-K_2O was applied at 100-80-50 kg ha^{-1}. *Bradyrhizobium japonicum* USDA 110 was used for the nodulation of soybean for all treatments in these experiments. The two PGPRs which increase the growth of pepper and cucumber seedlings were isolated from a plastic film house area in Korea by using the PBY medium. The isolates were identified as *Bacillus substilis* and *Bacillus megaterium* by the method of the biological test using API kit (BioMerieux Co., France; Table 1) and fatty acid analysis (MIDI; data not shown), and named as *B. substilis* SU-12 and *B. megaterium* SU-13. The used strains in these experiments were *B. subtilis* SU-12 (BS) and *Serratia proteamaculans* ATCC35475 (SP), both of which improve plant growth or yield in plant.

Inoculant preparation: *B. japonicum* used for the nodulation of soybean was cultured in a yeast extract mannitol culture medium (YEM)[17]. Broth was inoculated with slant material and cultured on an orbital shaker at 150 rpm for 7 days at 28°C. A subculture was prepared by inoculating new broth medium with the initial culture such that the added inoculant material constituted 1% of the volume of the subculture. Two PGPR strains (BS and SP) were cultured in LB medium and incubated on an orbital shaker at 150 rpm for 36 h at 30°C. Seven days after inoculation with *B. japonicum* and 2 days after inoculation with the PGPR strains, the cell density of *B. japonicum* and PGPR bacterial broth was diluted with new YEM and LB medium to $A_{620} = 0.2$[16]. The diluted *B. japonicum* bacterial broth was divided into four sterilized flasks, each flask representing one treatment. The cells in cultured bacterial broth were collected by centrifugation at 2,822 x g for 15 min at 4°C. The cells were adjusted to 1.0 x 10^9 cells mL^{-1}, based on optical density[16].

Plant growth: Soybean seeds (*Glycine max* Merr., cv. Gwangan) were surface-sterilized in 2% sodium hypochlorite for 3 min, and rinsed 5 times with distilled water[16]. Seeds were placed in sterilized vermiculite to germinate. Seven days after seeding, at the VE stage, seedlings were transplanted into sterilized pots (17 cm diameter and 15 cm deep) containing 2 kg of sterilized soil for 2 hr at 130°C, one seedling per pot. The PGPR effect on salinity levels was investigated by using 2 salinity levels (1.5 and 5.0 dS m^{-1}). Saline solution was applied only once at the beginning. The pots with the salinity treatment were equilibrated for 7 days before transplanting seedlings. A sterilized vinyl bag was put underneath each pot to collect excess water due to drainage. This water was reapplied to the respective pot. Three days after transplanting, one seedling was inoculated with 1 mL of inoculum containing approximately 10^8 cells[16]. *B. japonicum* strain was used in all treatments including the controls. The temperature in the greenhouse was maintained at 27 ± 2°C with a relative humidity of 65% and a 17 hr photoperiod created by using supplemental lighting from high-pressure sodium lamps. All plants were harvested 30 days after transplanting. The photosynthesis of plants was measured using a Li-Cor 6400 (Li-Cor Inc, Lincoln, Nebraska, USA) before harvesting the plants. To analyze antioxidative enzymes, fresh leaves were harvested 30 days after transplanting and then stored immediately into a deep-freezer (-80°C). The experiment was structured following a randomized complete block design (RCBD) with four replications.

Inorganic elements: Leaf tissues were separated after harvesting and air-dried at 70°C for 5 days. Dried materials were ground and then digested in H_2SO_4 for the determination of total nitrogen (Kjeldahl method[18]) or in a ternary solution (HNO_3:H_2SO_4:$HClO_4$ = 10:1:4 with volume) for the determination of P, K, Ca and Na[18].

Antioxidant activity: Malondialdehyde (MDA) content was assayed as an indicator of the extent of lipid peroxidation in leaf tissues by the method of Halliwell and Gutteridge[19]. MDA concentration was calculated by subtracting the absorbance at 535 nm from the absorbance at 600 nm using a molar extinction coefficient 1.56 x 10^5 M^{-1} cm^{-1}. Proline content in leaves was determined by colorimerty[20]. The H_2O_2 levels of ascorbate peroxidase (APX)[21] and glutathione reductase (GR)[22] in leaves were measured colorimetrically following the procedure described by Anderson[23]. The oxidation rate of ascorbate was estimated by following the decrease in absorbance at 290 nm for 3 min. Protein contents were determined according to the Bradford[24] method using bovine serum albumin (BSA) as a standard.

Statistical analysis: All data were analyzed statistically by an analysis of variance using CoStat software (CoHort Software, Monterey, USA). Salinity and PGPR treatments

Table 1: Biochemical test of the isolated bacteria by API kit

Nutrient	Bacteria		Nutrient	Bacteria		Nutrient	Bacteria	
	SU-12	SU-13		SU-12	SU-13		SU-12	SU-13
PB	-		SOR	-	-	GAL		+
BAC	-		SUC	-	+	AMG		-
OPT	-		TRE	-	+	MAL		+
HCS	-		ARA	-	+	PLA		-
6NC	+		PYR	-		NAG		+
10B	-		PUL	-		AMY		+
40B	-		INU	-	+	KCN		+
ESC	-	+	MEL	-		NCL		-
ARG	-		MLZ	-		MEN		+
URE	+		CEL	-		OLD		-
TZR	-	-	RIB	-	-	NAA		-
NOV	-		XYL	-	+	ARB		-
DEX	-		CAT	+		PAS		-
LAC	-		COAG	-		NAE		-
MAN	-	+	INO			THRM		-
RAF	-	-	GUL		+	TAG		-
SAL	-	-	NE6		-			

were tested in an experiment using a randomized complete block model with four replications. Mean comparisons were conducted using an ANOVA protected least significant difference (LSD) ($P<0.05$) test.

RESULTS AND DISCUSSIONS

Plant growth: To alleviate the negative effect of soil salinity on soybean physiological responses we co-inoculated *B. japonicum* with two PGPR strains, *B. substilis* and *S. proteamaculans*. Results of the measurement of growth response and photosynthetic rate are given in Table 2. Shoot and total dry weight under non-salinity conditions were significantly increased by all PGPR treatments. Total dry weight in all treatments containing PGPR strains under non-salinity stress was increased by 10.9-11.0%. Under salinity stress it also increased by 3.5-4.5% compared to the control treatment. Leaf area was increased under both salinity and non-salinity stress. PGPR inoculation increased leaf area by 6.5-10.1% for the non-salinity condition and 4.0-6.3% for the salinity condition compared to the control treatment. However, leaf length and leaf greenness were not significantly different between treatments. Photosynthetic rate was significant under non-salinity stress but under salinity stress it was not significantly increased. Photosynthetic rate in all treatments containing PGPR was increased by 3.9-6.3% compared to the non-salinity stress condition. The reduction of plant growth caused by salinity stress is the most common phenomenon of plants under stress, although the measurement of stress indicators might not be significant. Marketable yield was significantly reduced in the high salinity treatment compared to the control. In addition, the high salinity treatment with *B. subtilis* had an even lower yield despite improved vegetative plant growth[25]. A similar result was reported by Vivas et al.[26] who showed that inoculation with *Bacillus* sp. and coinoculation of it with *Glomus* sp. both increased stomatal conductance of lettuce compared to a non-drought control. In this study, inoculation with PGPR strains increased plant growth compared to the non-inoculated control treatment, and the inoculation with PGPR strains under soil salinity conditions improved plant growth compared to the non-inoculated control.

Mineral content: The PGPR strains varied greatly in their effect on the concentration of major mineral nutrients in soybean leaves under soil salinity conditions (Table 3).

Table 2: PGPR effect on yield, photosynthetic rate and growth of soybean seedlings grown for 4 weeks under soil salinity conditons

Salinity (dS m^{-1})	Strain	Dry weight (g $plant^{-1}$)			Leaf area (cm^2 $plant^{-1}$)	Plant height (cm)	Leaf greenness (SPAD)	Photosynthetic rate (umol CO_2 m^{-2} s^{-1})
		Shoot	Root	Total				
1.5	Control	0.976b	0.165	1.141b	277b	34.6	30.7	12.7b
	BS	1.094a	0.171	1.266a	303a	34.5	31.2	13.3a
	SP	1.092a	0.175	1.267a	295a	34.2	31.3	13.2ab
	BS+SP	1.090a	0.176	1.266a	305a	33.0	31.7	13.5a
5.0	Control	0.681	0.121	0.802b	175b	25.5	15.8	8.7
	BS	0.708	0.127	0.835a	186a	25.1	16.6	9.4
	SP	0.709	0.129	0.838a	182ab	25.0	16.8	9.1
	BS+SP	0.702	0.128	0.830a	186a	24.6	16.7	9.4
Significance of factors	Salinity	***	***	***	***	***	***	***
	Strain	**	ns	**	**	ns	ns	**
	Interaction	*	ns	ns	ns	ns	ns	ns

*, ** and *** significant at the 95%, 99% and 99.9% confidence level and ^{ns} is not significant
BS, *Bacillus subtilis*; SP, *Serratia proteamaculans*

Table 3: PGPR effect on mineral uptake of soybean seedlings grown for 4 weeks under soil salinity conditions

Salinity (dS m^{-1})	Strain	N (mg $plant^{-1}$)	P (mg $plant^{-1}$)	K (mg $plant^{-1}$)	Ca (mg $plant^{-1}$)	Na (mg $plant^{-1}$)
1.5	Control	24.8	5.9b	52.7b	14.3b	3.6a
	BS	26.6	6.6a	60.8a	16.0a	3.5a
	SP	26.6	6.7a	60.3a	16.3a	3.5a
	BS+SP	26.3	6.6a	61.1a	16.4a	2.8b
5.0	Control	16.4	3.8b	25.4a	7.5	23.1a
	BS	17.1	4.1a	24.9a	8.2	22.1b
	SP	16.6	4.1a	25.1a	7.9	23.0a
	BS+SP	17.1	4.1a	22.2b	8.9	22.7a
Significance of factors	Salinity	***	***	***	***	***
	Strain	ns	**	**	**	**
	Interaction	ns	ns	ns	ns	ns

*, ** and *** significant at the 95%, 99% and 99.9% confidence level and ^{ns} is not significant
BS, *Bacillus subtilis*; SP, *Serratia proteamaculans*

The N, P, K, Ca and Na uptake per plant in the soil salinity treatment were significantly decreased compared to the non-salinity treatment. An interaction between salinity and the strains was not found. The concentration of major cations in the non-salinity treatment was increased more with the PGPR treatment than the control without PGPR strains, but K^+ and Na^+ uptake under soil salinity were decreased by 1.2-11.8% and 0.4-4.3%. Cation uptake under soil salinity was investigated by a co-inoculation treatment (*B. substilis* plus *S. proteamaculans*). The *S. proteamaculans* strain caused a lower uptake of K and Na compared to *B. subtilis*. This means that the *B. subtilis* strain could alleviate the effects of salinity stress in soybean. These PGPR strains can produce bacterial exopolysaccharides (EPSs) that bind cations, including Na^+[14]. It may postulated that increasing the population density of EPS-producing bacteria in the root zone could decrease the content of Na^+ available for plant uptake, thus helping to alleviate salt stress in plants[15]. Inoculation restricted Na^+ uptake by the roots, especially in the inoculated plants compared to the un-inoculated plants. This was probably caused by a reduced passive

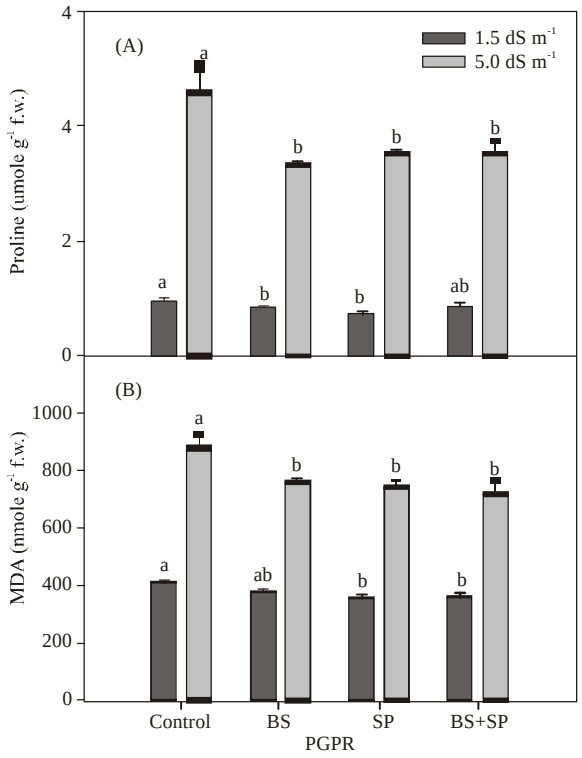

Fig. 1: PGPR effects on proline and MDA activity of soybean leaves under soil salinity. Means with the same letter are not significantly different at P<0.05 when compared by LSD. Treatment means are with ±S.E. of four replications. BS, *Bacillus subtilis*; SP, *Serratia proteamaculans*

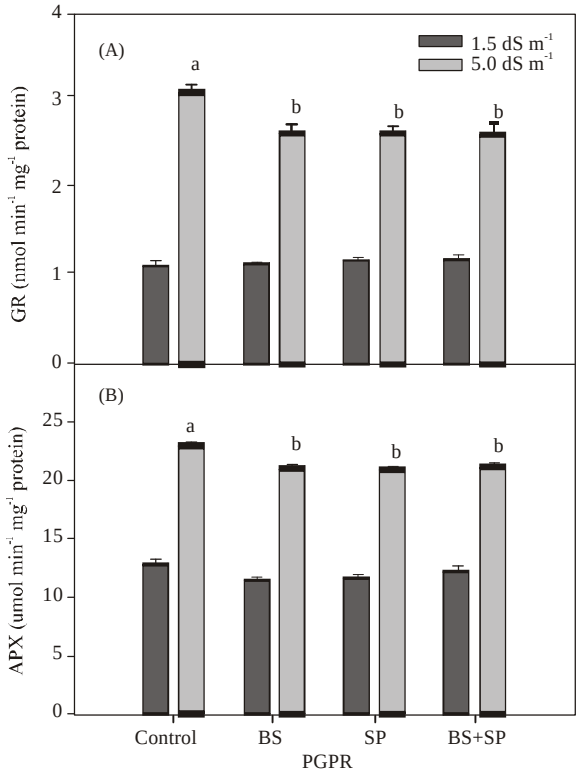

Fig. 2: PGPR effects on GR and APX activity of soybean leaves under soil salinity. Means with the same letter are not significantly different at P<0.05 when compared by LSD. Treatment means are with ±S.E. of four replications. BS, *Bacillus subtilis*; SP, *Serratia proteamaculans*

(apoplasmic) flow of Na^+[15]. *B. subtilis*, which is well known for increasing EPS production, along with the co-inoculation treatments were the most efficient, whereas *S. proteamaculans* was less effective. The results suggested that inoculation with selected EPS-producing bacteria could serve as a useful tool for alleviating salinity stress in salt-sensitive plants.

Antioxidant activity: Salt-stressed plants accumulated various organic compounds such as proline, glucose, glycine betaine etc. in the cell membrane for osmoregulation to occur to enable growth by protecting enzyme activity[27]. However, to understand the protective action of antioxidants against salinity stress, we treated with PGPR strains and then measured the level of antioxidant activity. The results are presented in Figures 1 and 2. The un-inoculated plants, compared to the inoculated plants, under soil salinity conditions had an increased antioxidant activity and concentration of proline, MDA, GR and APX. The results suggested that inoculation of salt-stressed plants with PGPR strains could alleviate salinity stress. Bacterial exopolysaccharides (EPSs) in the soil ecology system play an important role in soil aggregation[28] and soil adhesion[29]. EPS-producing bacteria under soil salinity conditions have been found to restrict Na^+ uptake by wheat roots[15]. However decreased Na^+ can decrease antioxidant levels. PGPR strains, especially EPS-producing bacteria, can induce soil salinity tolerance and growth promotion in soybean seedlings under greenhouse conditions.

REFERENCES

1. Singleton, P.W. and B.B. Bohlool, 1984. Effect of salinity on the nodule formation by soybean. Plant Physiol., 74: 72-76.
2. Lauter D.L., D.N. Munns and K.L. Clarkin, 1981. Salt response of chickpeas influenced by N supply. Agronomy J., 73: 961-966.
3. Drew, M.C., P.S. Hole and G.A. Picchioni, 1990. Inhibition by NaCl of net CO_2 fixation and yield of cucumber. J. Amer. Soc. Hort. Sci., 115: 472-477.

4. Hasegawa, P.M., R.A. Bressan, J.K. Zhu and H.J. Bohnert, 2000. Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol., 51: 463-499.
5. Parida, A.K. and A.B. Das, 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxic. Environ. Safety, 60: 324-349.
6. Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci., 7: 405-410.
7. del Rio, L.A., F.J. Corpas, L.M. Sandalio, J.M. Palma and J.B. Barroso, 2003. Plant peroxisomes, reactive oxygen metabolism and nitric oxide. IUBMB Life, 55: 71-81.
8. Smirnoff, N., 1993. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol., 186: 69-74.
9. Noel, T.C., C. Sheng, C.K. Yost, R.P. Pharis and M.F. Hynes, 1996. *Rhizobium leguminosarum* as a plant growth-promoting rhizobacterium: direct growth promotion of canola and lettuce. Can. J. Microbiol., 42: 279-283.
10. Kloepper, J.W. and M.N. Schroth, 1978. Plant growth-promoting rhizobacteria on radishes. IV. International Conference on Plant Pathogenic Bacteria. Angers France, 2: 879-882.
11. Woitke, M., H. Junge and W.H. Schnitzler, 2004. *Bacillus subtilis* as growth promotor in hydroponically grown tomatoes under saline conditions. Acta Horticulturae, 659: 363-369.
12. Saleh, S.A., H. Heuberger and W.H. Schnitzler, 2005. Alleviation of salinity effect on artichoke productivity by *Bacillus subtilis* FZB24, supplemental Ca and micronutrients. J. App. Bot. Food Qual., 79: 24-32.
13. Dashti, N., B. Prithiviraj, X. Zhou, R.K. Hynes and D.L. Smith, 2000. Combined effects of plant growth-promoting rhizobacteria and genistein on nitrogen fixation in soybean at suboptimal root zone temperatures. J. Plant Nut., 23: 593-604.
14. Geddie, J.L. and I.W. Sutherland, 1993. Uptake of metals by bacterial polysaccharides. J. App. Bacteriol., 74: 467-472.
15. Ashraf, M., S.H. Berge and O.T. Mahmood, 2004. Inoculating wheat seedlings with exopolysaccharide-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. Biol. Fer. Soils, 40: 157-162.
16. Bhuvaneawari, T.V., B.G. Turgeon and W.D. Bauer, 1980. Early events in the infection of soybean (*Glycine max* L. Merr.) by *Rhizobium japonicum* I. location of infectable root cells. Plant Physiol., 66: 1027-1031.
17. Vincent, J.M., 1970. A manual for the practical study of the root-nodule bacteria. Burgess and son Ltd., Great Britain, pp 45.
18. RDA (Rural Development Administration, Korea), 1988. Methods of Soil Chemical Analysis. National Institute of Agricultural Science and Technology, RDA, Suwon, Korea.
19. Halliwell, B. and J.M.C. Gutteridge, 1989. Free Radicals in Biology and Medicine. 2nd Eds. Oxford, Clarendon Press, UK.
20. Bates, L.S., R.P. Waldren and I.D. Teare, 1973. Rapid determination of free proline for water stress studies. Plant Soil, 39: 205-207.
21. Chen, G.X. and K. Asada, 1989. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol., 30: 987-998.
22. Rao, M.V., G. Paliyath and D.P. Ormrod, 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol., 110: 125-136.
23. Anderson, J.A., 2002. Catalase activity, hydrogen peroxide content and thermotolerance of pepper leaves. Sci. Hort., 95: 277-284.
24. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
25. Woitke, M., A. Hanafi and W.H. Schnitzler, 2004. Effect of salinity and *Bacillus subtilis* on white fly (*Trialeurodes vaporariorum*, Westwood) in hydroponically grown tomatoes (*Lycopersicon esculentum* Mill.). Acta Horticulturae, 659: 323-329.
26. Vivas, A., A. Marulanda, J.M. Ruiz-Lozano, J.M. Barea and R. Azcon, 2003. Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. Mycorrhiza, 13: 249-256.
27. Munns, R. and A. Termaat, 1986. Whole-plant responses to salinity. Aust. J. Plant Physiol., 13: 143-160.
28. Chenu, C. and J. Guerif, 1991. Mechanical strength of clay minerals as influenced by an absorbed polysaccharide. Soil Sci. Soc. Am. J., 55: 1076-1080.
29. Bezzate, S., S. Aymerich, R. Chambert, S. Czarnes, O. Berge and T. Heulin, 2000. Disruption of the *Paenibacillus polymyxa* levansucrase gene impairs its ability to aggregate soil in the wheat rhizosphere. Environ. Microbiol., 2: 333-342.