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RHIZOBIA STRAINS ALLEVIATE SALINITY IN FABA BEANS (*VICIA FABA*) TO VALORIZE MARGINAL SOLS

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Abstract

Soil salinity remains the most significant limiting factor for the growth of *Vicia faba L* (faba bean) in Tunisia. Plant growth-promoting rhizobacteria (PGPR) has emerged as a novel way of minimizing the harmful effects of salt stress and improving nutrient accessibility. The aim of this investigation is to study the protective effects of two newly isolated PGPR (rhizobia strains S1 and S2) over 150 mm NaCl salinity stress. Physiological & biochemical parameters along with antioxidant enzymes activities in faba bean culture were measured. The Results showed that salt stress significantly increased nodule biomass in plants inoculated by S1 as compared to S2. Salinity augmented the level of nitrogen in root and shoot in case of S1, while no change was observed with S2 inoculation. Similarly, higher level of potassium and sodium was observed in plants inoculated with S1 compared to S2. Electrolyte leakage (EL) showed an increase of 80% in leaves of plant inoculated with S_2 150 mM NaCl against 10.71 % in leaves of plant inoculated with S_1 after 10 days of inoculation. After 30 days of inoculation under salinity faba bean-S1 showed a higher value of PPO (8.86 µmol g^{-1}FM) and without salt, the PPO activity was 2 µmol g^{-1}FM. After 40 to 50 days of inoculation, plants inoculated by S1 and S2 showed higher values of phenol content under salinity. The results indicated that salinity significantly increasesd proline accumulation in the leaves and root of faba bean inoculated with S1. However, chlorophylls level was not affected. This study indicates that the S1 rhizobia strain is a prospective inoculant candidate which is essential for promoting growth and production, and thereby reducing the effect of salinity on faba bean plant.

Key words: Salt stress, Rhizobacteria, PGPR, Rhizobia, *Vicia faba*, Oxidative stress.

Introduction

Faba bean (*Vicia faba* L.) is an important leguminous crop and is cultivated in maximum parts of the world for human consumption. Faba bean (*Vicia faba* L.) is an important leguminous crop and is cultivated in maximum parts of the world for human consumption (Hashem *et al*., 2012). *Vicia faba*, a major protein crop occupies a large area of cultivated land in Tunisia (Kharrat *et al*., 1991). Major reasons for cultivation of crop include, (1) diversification of agricultural areas resulting in decreased disease as well as weed and pest build-up and ameliorates biodiversity, (2) optimizing the consumption of fossil energy in plant production, (3) providing protein rich food and (4) its ability to supply biologically fixed nitrogen (N) to the system (Erik *et al*., 2010). *Vicia faba* cultivation also has a major benefit of increasing the nitrogen content of soil by its symbiotic association with rhizomes (Hungria & Vargas 2000). Legume culture is drastically affected by cold stress, low nutrient accessibility and drought. A major blowout is brought about by salinization of the cultivated land (Abdi *et al*., 2012). Salinity induced abiotic stress remains a nightmare (Zheng *c*) causing serious environmental problems and reduced agricultural productivity (Ying *et al*., 2012). Nearly 50% of cropland and 20% of the cultivated areas have already been lost due to higher salt level (Lakhdar *et al*., 2009). Even more alarming is the forecast that above 50% of major arable land will be vulnerable to salinization around the year 2050 (Wang *et al*., 2009). Biotechnological approaches have contributed in the increase of crop production in salinity affected areas (Zahir *et al*., 2004). One such example is root-colonizing nonpathogenic rhizobacteria which can improve the resistance of the plant against abiotic and biotic constraints (Yang *et al*., 2010) and enhance soil fertility and plant growth (Rabie & Almandini, 2005). Implementing stress-tolerant microbial strains related to agronomic crops roots (especially rhizobia strains with legumes) may improve plant adaptability to severe climatic conditions and soil fertility (Wu *et al*., 2009). Bacteria surviving under extreme environmental conditions are useful for various agricultural activities (Egamberdieva & Kucharova, 2009). The interaction between microorganism and legumes that can affect a plant's environmental resistance occur in the soil (Siddikee *et al*., 2010). At present, the greatest importance resides in developing and applying trait-specific bacteria inoculants that could improve stress tolerance in plants (Neelam & Meenu, 2010) and disease management (Whips, 2001). Considering the decay in yield because of the severity of abiotic constraints, particularly salt stress, the idea of plants becoming more resistant to stress through the use of bacteria inoculant has gained popularity (Shweta *et al*., 2011). In this respect, the bacteria surviving in high salinity areas can play the role of potential inoculants for plant productivity (Shweta *et al*., 2011). The objective of this research is to study the

influence of salt-tolerant rhizosphere microbe (rhizobia strain) in relation to stress tolerance. The study provides new concepts to enhance our knowledge regarding plants stresses tolerance mechanism through bacteria inoculation.

Materials and Methods

The seeds of faba bean *L. cv. Bachar,* widespread in Tunisian agriculture were separately inoculated with two bacteria (S1, S2) which had a high tolerance to salt. Mhamdi *et al*., (2000) reported that faba bean cultivated in Tunisian area were nodulated by rhizobia belonging to 7 species of the genera *Rhizobium* and *Sinorhizobium* as recognized by analysis of 16S ribosomal gene. Seedlings were cultivated under adapted conditions. They were sterilized in 2% $Ca(ClO)_2$ then washed with distilled H_2O and finally placed for germination at around 29°C in media consisting of hundred ml volume of Bergersen solution (Vincent, 1970). The bacteria introduced were synthesized from rhizobia which were well known for its high tolerance to salt and conserved at 4°C on YEM medium (Vincent, 1970). Liquid YEM was used for propagation of bacteria under agitation for 48 hours at 29°C in obscurity. Inoculation was realized from 4-day-old seedlings which were soaked for half an hour in100 ml solution containing roughly 10^8 cells /ml. The seedlings were transferred into pots which were filled with one kg of the uncontaminated perlite. Subsequently, the pots were divided into 2 sets (4 plants for each treatment) depending on the salt content (0 and 150 mM of NaCl). Both the sets were then irrigated with a similar nutrient solution. Plants were harvested at a flowering period of culture and roots were separated having nodules from the shoots. Plants were dehydrated at 70ºC for 48 hours until the dry mass was stabilised. Dry mass was then measured.

Evaluation of the contents of nitrogen and phosphate in roots and shoots: Roots and shoots of phosphate were evaluated using molybdate blue technique as explained by Murphy & Riley (1962). A mass of 0.3 g of dehydrated matter was calcined and filtrated using Whatman filter and to the final volume, water was added to adjust the volume at 50 ml. 100 µl by volume of each extract was introduced to the reactive mixture (2.5 ml) which was composed of hydrazine sulfate (2.5%) and ammonium molybdate (0.15%). Subsequently, absorbance was measured at 820 nm using room temperature following incubation for 30 mins. 0.5 g root and shoot samples were analyzed by the Kjeldahl method to determine the nitrogen level.

Evaluation of the contents of potassium and sodium in roots and shoots: The dry biomass (0.3g) was calcinated and filtrated and the solution was adjusted to 50 ml by adding ionized water. Potassium$^+$ and sodium content was estimated using a spectrophotometer. The data were expressed in mg by Kg of dry matter.

Electrolyte leakage (EL): (EL) was employed to evaluate the stability of the cell membrane. The EL and the electrical conductivity are related to the EC (Ghoulam *et al*., 2002). The Electrolyte Leakage is expressed in EL (%) = (EC1/EC2)*100, where EC1 and EC2 denote the obtained values from incubated leaves at 25°C for one day and that measured for autoclaved leaves at 120°C during 20 min, respectively.

H_2O_2 ratio in leaves and nodules: H_2O_2 content of leaves was determined following Velikova *et al*., (2000). A quantity of 100mg of collected leaves was grounded and mixed with 2 ml of TCA (20%) and then centrifuged at 4°C for 15 minutes. The H_2O_2 content in these extracts was determined. 0.5 ml of the extract was mixed with an equal volume of buffer and 1ml of K iodine (1M) was then measured at 390 nm after 24h of incubation. The H_2O_2 amount was expressed in µmol per gram of fresh biomass referring to a standard that had been prepared under similar conditions and known H_2O_2 concentrations.

The peroxidase (PO) and polyphenol oxidase (PPO) extractions: PO and PPO were extracted from homogeneous mixture of 100 mg of leaves containing 10% per weight of polyvinyl poly pyrrolidone and 1ml of buffer at 4°C. The obtained homogenates were then centrifuged at 4°C for 20 min. The supernatants were analyzed in order to estimate the activity of PO and PPO (Tejera *et al*., 2004). The activities of peroxidase were measured (Diani *et al*., 2009). The reaction mixture used consisted of 200 µL of H_2O_2 at 0.3%, 300 µL of guaiacol at 20 mM, 2 mL of buffer, 1 mL of H_2O and 50 µL of the extract of enzyme. The activity of peroxidase was estimated by monitoring the decomposition of H_2O_2 at 470 nm. The activity of polyphenol oxidase was measured (Hori *et al*., 1997). The reaction mixture consisted of 500 µL catechol at 1.6 % in a buffer, 250 µL of H_2O, 200 µL of buffer and 100 µL of the extract of enzyme and absorbance was measured at 410 nm. The optical density was determined every 60s during 3 min of incubation time. Distilled water was taken as reference. PPO activities were evaluated as the quantity of protein that decomposes 1 µmol of H_2O_2 per g FW.

Total phenols content: The roots samples resulting from the various treatments were course blended using pre-cooled mortar pestle and subsequently extracted at 4°C with methanol (80%) under permanent agitation. The obtained homogenate was centrifuged for 3 minutes and then each supernatant was measured with a spectrophotometer. The total phenols content was calculated according to Foline-Ciocalteu technique (Dicko *et al*., 2002). The absorptions were recorded on Cary100-UV spectrophotometer at 760 nm. A calibration line was traced based on freshly prepared (+)-catechin solutions. Phenol contents were expressed as mg equivalent (+)-catechin per g of fresh weight (FW).

Contents of proline and chlorophylls: Proline content was measured by spectrophotometry (Bates *et al.,* 1973). B From 100 mg fresh matter of roots, nodules and leaves, proline was extracted, with 2 ml of methanol (40%). The temperatures of the tubes were fixed at 85°C in an H_2O bath for half an hour. 1 ml of extract was mixed with orthophosphoric acid (6 M), 1ml of glacial acetic acid and 25 mg ninhydrin. Each tube

was cooled to room temperature after incubation for 1 hour at 100°C. 5ml of toluene was supplied after that. The absorbances of the upper phase were measured at 528 nm using spectrophotometer whereas the concentration of proline was measured using a typical line traced by means of solutions of proline with known concentration. Each Concentration of chlorophyll a and b were determined by the subsequent equations (Lichtenthaler & Wellburn, 1983):

$$\text{Chl a} = (11.75*A_{562})-(2.35*A_{645})$$
$$\text{Chl b} = (18.61*A_{545})-(5.03*A_{662})$$

Results

Nodulation and plant growth: Nodulation varied, depending on the effect of salinity and the tested strain. Under optimal conditions, nodulation was 7 and 90 nodules per plant inoculated with S1 and S2 respectively. However, under salt stress, the number varied significantly and differently in response to S1 and S2 inoculation (Fig. 1). Nodulation was increased by 92% in plants inoculated with S_1 while it was decreased by 58% in plant inoculated with S2 (Fig. 1). Increased nodule biomass was observed under salt stress. This increase was significant for plants inoculated with S1, whereas insignificant changes were observed in plants inoculated with S2. Results showed that the salt didn't significantly affect the shoot dry matter of all tested Faba bean-rhizobia symbioses. Shoot dry weight was in the order of 0.6g/Pl. (Fig. 2A). Overall, the salt decreased root dry weight of plants inoculated by S1 by approximately 49.33%. On the other hand, salinity in the plant inoculated by S2 did not affect root dry weight of *Vicia faba*-rhizobia symbiosis (Fig. 2B).

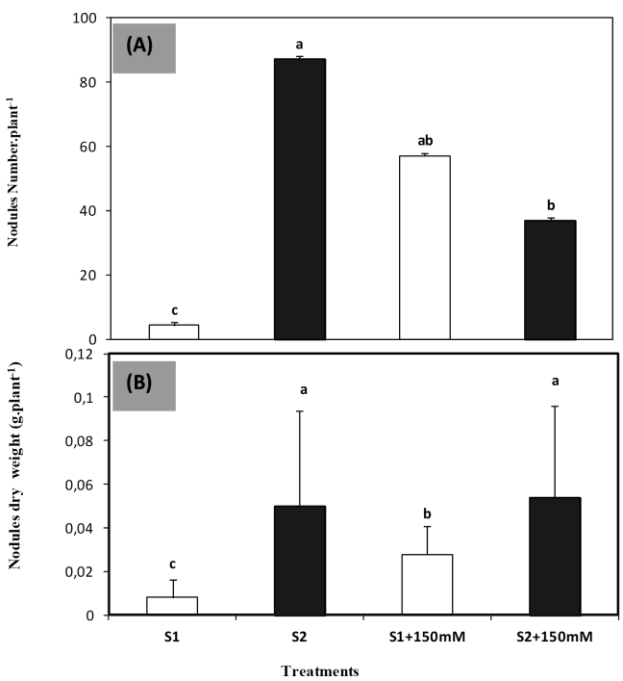

Fig. 1. Number of nodules (A) and their dry weight (B) of faba bean-rhizobia combinations grown under optimal conditions (S_1 and S_2) and under salt stress (150mM). Average and SE of five replicates harvested at flowering stage.

Shoot and root N content: Nitrogen content varied differently according to rhizobia strain, plant shoot / root and culture conditions. When the two rhizobial strains were compared under salt stress, the shoot and root N content were increased in plant inoculated with S1 more than in plant inoculated with S2 (Fig. 3).

The contents of phosphate, potassium and sodium in Root and shoot: Results indicate higher increase of phosphorus contents by salt stress in root and shoot for faba bean-S1 which was 28.19% and 66.10% respectively (Table 1). In contrast, for the plants inoculated with S_2, the salinity increased the phosphorus content in the root but decreased it in the shoot. Shoot and root potassium and sodium content of symbiotic combinations showed significant increase at the same time. Under 150 mM NaCl, K^+ and Na^+ uptake by the shoot of plant inoculated with S1 was increased by 19.28% and 39.79% respectively. The same trend was observed in the roots of plant inoculated with S1. However, potassium and sodium did not change significantly in plant inoculated with S_2 (Table 1).

Electrolyte leakage: Time-dependent variation in EL of faba bean symbioses was induced by the salt stress. It was noted that the highest level of EL under salt stress was observed after 10 days of inoculation by S1 and S2 strain which was significantly declined after 20 days. Results also showed that after 40 days, EL decreased in leaves of all treatments (Fig. 4). Generally, EL values were decreased by salt in leaves of plant inoculated with S2 and S1 under 150 mM NaCl concentration. In contrast, an increase of 80% was observed in leaves of plants inoculated with S2 compared to 10.71% in plants inoculated with S1 after 10 days of inoculation.

Content of hydrogen peroxide: Under salinity, the content of hydrogen peroxide was increased significantly in the leaves, depending on time (Fig. 5). Significant differences in the hydrogen peroxide content were noted after 30 days of inoculation, which further reached the highest peak content after 50 days of inoculation (Fig. 5). The observed value of H_2O_2 content in leaves after 50 days was six times higher compared to the values recorded after ten days.

Activities of peroxidase and polyphenol oxidase: Significant lower variation in activity of PO in leaves was observed over time. At 30 and 40 days of inoculation, salinity increased PO by 4 µmol g^{-1}FM with all treatments. However, this activity was declined at 20 and 50 days of inoculation. In general, salt stress did not affect PO activity (Fig. 6A). However, higher PPO activity was observed over time compared to the expected value of 10 µmol g^{-1}FM. After 30 days of inoculation under salinity, faba bean-S1 showed a higher value of PPO (8.86 µmol g^{-1}FM) and without salt, the PPO activity was 2 µmol g^{-1}FM (Fig. 6B). Salt stress stimulated polyphenol oxidase activity in leaves compared to peroxidase.

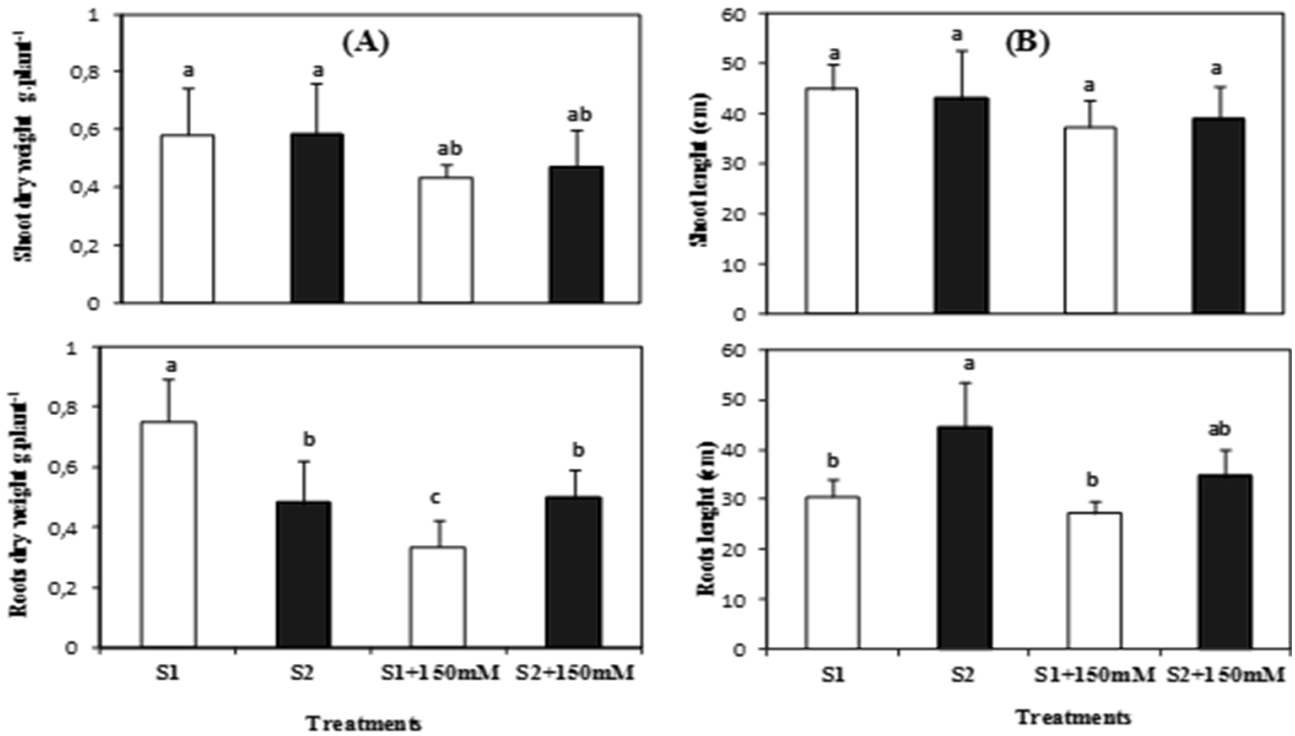

Fig. 2. Dry weight of root & shoot (A) and length (B) of faba bean-rhizobia cultivated under salt stress (150mM). Means and SE of five replicates harvested at flowering stage.

Table 1. The content of elements (P, Na, K) in shoot and root of faba bean plant inoculated by two rhizobia strain (S1and S2) grown under salt stress (150mM). Values are means and SE of four variations harvested at flowering stage.

	Shoot			Root		
	P (mg/Kg DW)	K (mg/Kg DW)	Na (mg/Kg DW)	P (mg/Kg DW)	K (mg/Kg W)	Na (mg/Kg DW)
S1	1,32 ± 0,03	21,60 ± 0,01	19,97 ± 0,03	0,24 ±0,06	4,95 ± 0,13	24,95 ± 0,05
S2	1,91 ± 0,03	23,13 ± 0,01	18,28 ± 0,12	0,60 ± 1,31	9,69 ± 0,02	31,52 ± 0,02
S1+150	2,61 ± 0,02	26,76 ± 0,02	33,17 ± 0,27	2,30 ± 0,33	11,29 ± 0,04	32,28 ± 1,41
S2+150	1,66 ± 0,01	26,70 ± 0,01	30,01 ± 0,37	1,77 ± 0,42	8,61 ± 0,14	31,28 ± 0,37

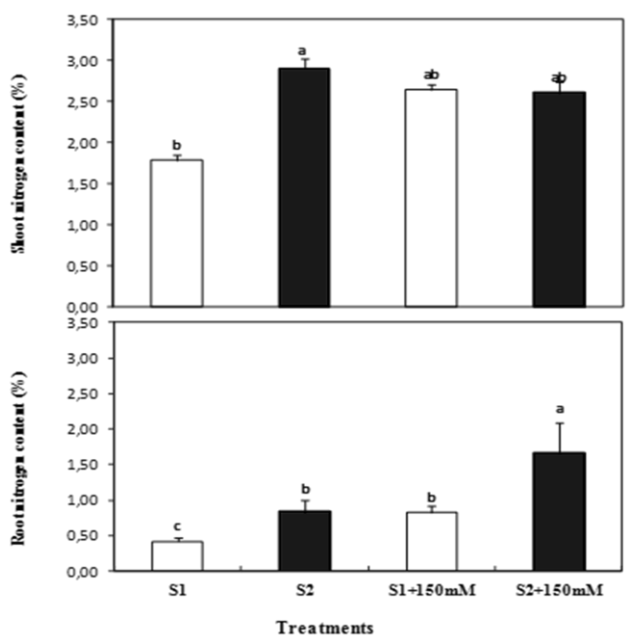

Fig. 3. Percentage of N in shoot and root of faba bean-rhizobia cultivated under salt stress (150mM). Data are means and SE of five replicates harvested at flowering stage.

Total level of phenol: The total level of phenol in symbioses was significantly improved by salinity (Fig. 7). A further increase of 14.7% was observed after 30 days of inoculation for all treatments. After 40 to 50 days of inoculation, plants inoculated by S1 and S2 showed higher values of phenol content under salt stress.

Effect on proline and chlorophyll content: The salt stress is responsible for enhanced accumulation of proline in the leaf of *Vicia Faba*-bacteria symbiosis inoculated with S1. However, this accumulation was decreased in nodules and the result indicated that salinity reduced the accumulation of proline in leaves and nodule with plant inoculated with S2 (Fig. 8). The content of proline in root was increased in plants inoculated with either S1 or S2 under 150 mM NaCl. Chlorophyll level showed no significant variation between different treatments (Fig. 9).

Discussion

Faba bean is vital for nitrogen cycling in agrionomy because of their symbiosis with the N-fixing rhizobia. Rhizobial strains are known to develop enzymatic profiles essential for the tolerance to abiotic stress (Zinjarde *et al.*,

2014) because they are usually exposed and adapted to environmental conditions. Use of bacterial inoculation to promote plant growth is well documented in the literature (Egamberdieva *et al*., 2011). In addition to the plant growth-promoting property, S1 also possess salt stress tolerance which makes it a potential candidate to alleviate salinity concerns in faba bean production. S1 seems to be the salt-tolerant and more efficient strain than the S2. S2 was observed to be highly sensitive to salt. The faba bean was judged as a potential candidate on the basis of its ability stabilize its shoot's dry weight under salt stress, and it was enhanced nodulation tolerance to salt in plant inoculated with S1 than with S2. In addition, inoculation with S1 was also showed the better activity of nitrogen fixation in shoot and root, expressed as nitrogen percentage on a dry weight basis. The increased in shoots, rather than roots, of N, P, K, Na was evidence of the adaptability of the above studied rhizobial symbioses. These points had been proved for different species such as rice, sorghum and soybean (Djanaguiraman *et al*., 2006). Under salinity, higher K^+ and Na^+ level were observed in plants inoculated with S_1 than in plant inoculated by S2. For the faba bean, S1 symbiosis had been referred to as physiological test indicator for salt stress tolerance in plants. The last test results were coincided with results of previous studies (Kaya *et al*., 2007). The unchanged effect of salinity stress on chlorophyll contents in faba bean- rhizobia symbiosis may be ascribed to its elevated content of antioxidants. It might be an adaptation for protection of the genotypes against chlorophylls alteration. In this respect, Yildirim *et al*., (2008) reported that chlorophylls level was a significant test of salinity in yield plant. Ghoulam *et al*., 2002 noticed that plant had developed complex mechanism permitting adaptation to ionic and osmotic stress which were induced by high salinity under salinity. The last mechanism comprises osmotic regulation by the collection of compatible solutes such as polyols proline and glycinebetaine (Yeo, 1998). The data indicated that the majority of tolerant faba bean-rhizobia accumulated higher proline content in leaves than in nodules and root. These results implied that this solute had an essential function in salt adaptation in *Vicia faba*. Some of the previous research reported higher proline concentration in salt-tolerant plants (Ashraf & Harris, 2004). Some studies indicate salt tolerance is intimately related to the amassing of proline in leaves (Farissi *et al*., 2011). The proline acts not just an osmolyte, but in addition helps the cell to overcome oxidative stress in salinity stressed plant (Rajendrakumar *et al*., 1994).

Under aforesaid conditions, S1 strain might have helped plants to evolve another complex mechanism favouring adaptation to ionic and osmotic stress indicated by salt stress (Ghoulam *et al*., 2002). Which includes adaptive accumulation of matched solutes such as PPO and phenols. Our results indicated that under salt stress, root of faba bean inoculated by rhizobia accumulated a higher content of phenols after 40 to 50 days of inoculation and after 30 days of inoculation under salinity faba bean- S1 showed the higher value of PPO. It may, therefore, be presumed that this solute could participate in salt tolerance in faba bean (Ashraf & Harris, 2004). Similarly, Abdi *et al*., (2012) found that level of phenols in root represents a type of plant resistance against abiotic stress.

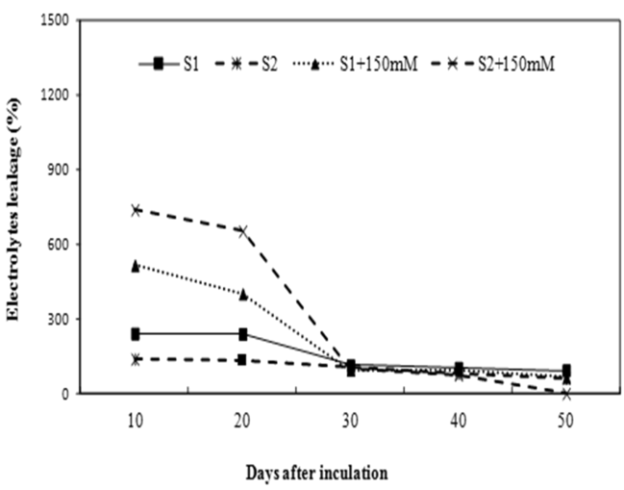

Fig. 4. Leakage of electrolytes in leaves of faba bean-rhizobia cultivated under salt stress (150mM). Mean and SE of four replicates harvested at different stages of culture.

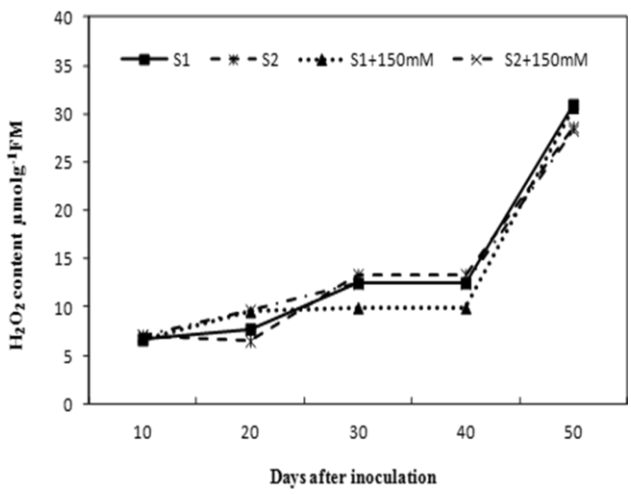

Fig. 5. H_2O_2 percentage in leaves of faba bean-rhizobia cultivated under salt stress (150 mM).

Conclusion

Overall observations from this study indicate that inoculation of rhizobial strains can protect faba bean against salinity. S_1 rhizobia strain is a prime candidate of an inoculant (PGRP), for improving plant development and reducing the effect of salinity on faba bean. Consequently, rhizobia strains especially S_1 is a candidate to be used as a bio-fertilizer and protect *Vicia faba* culture against salinity. Thus, a careful combination of rhizobia inoculants with optimized production measures such as resistant cultivars could provide good crops.

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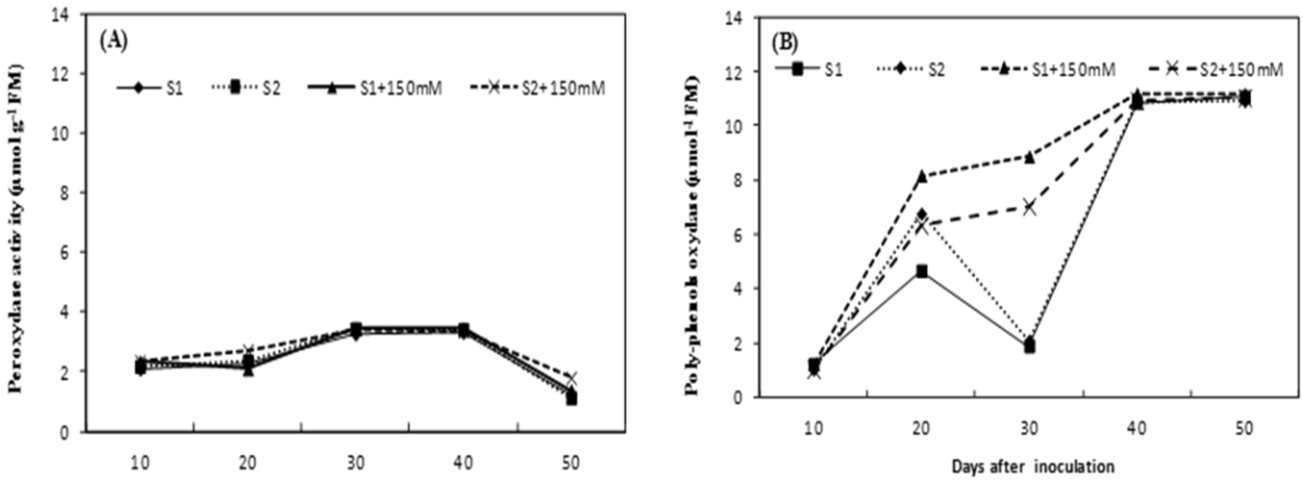

Fig. 6. Peroxidase and polyphenol oxidase activities in leaves of faba bean-rhizobia cultivated under salt stress supply (150mM). Data are means and SE of four replicates harvested at different stages of culture.

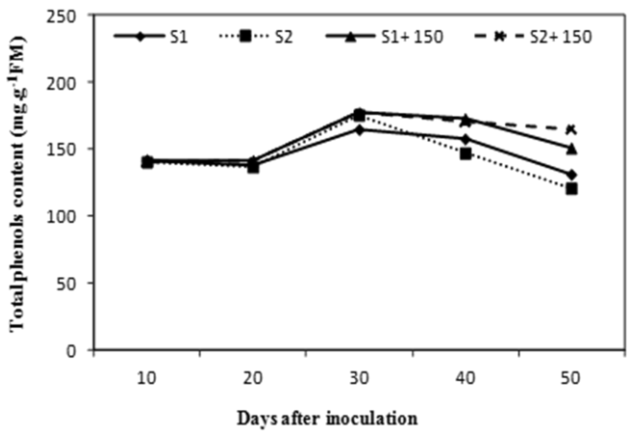

Fig. 7. Phenols in roots of faba bean-rhizobia cultivated under salt stress (150mM NaCl). Data reflects average and SE of four replicates harvested at 10, 20, 30, 40 and 50 days after inoculation by S_1 and S_2. Statistical analysis was done separately for each stage of culture.

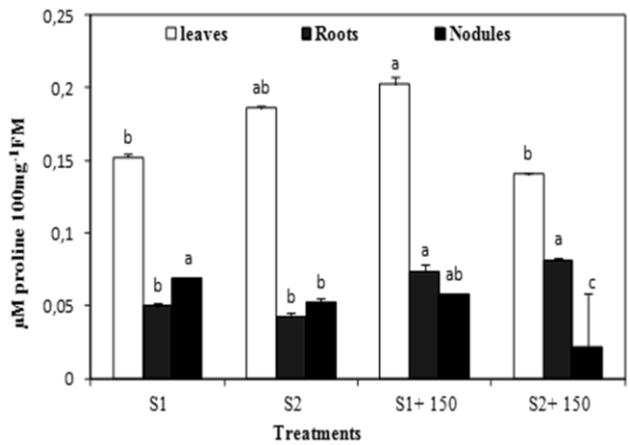

Fig. 8. Proline (µM.100 mg^{-1} FM) in the leaves, roots and nodules of faba bean-rhizobia symbiosis (150 mM NaCl). Data is average and SE of four replicates at the flowering stage.

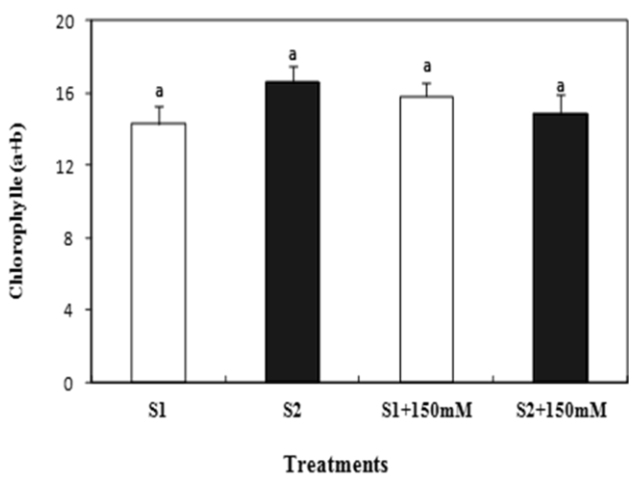

Fig. 9. Chlorophylls percentage in the leaves of faba bean inoculated by two rhizobia strains ($S_1$and S_2) under salt stress (150 mM NaCl).

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