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Effects of Salt Stress on Some Nitrogen Fixation Parameters in Faba Bean

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Abstract: The adverse effects of sea water salinity on number of nodules, nitrogen content, nitroginase activity, Chlorophyll a and b content, proline accumulation and protein pattern of faba bean plants (*Vicia faba* commercial cultivar Nubaria 1) were investigated. Faba bean plants were irrigated with sea water at 20, 25, 30, 40 and 50% concentrations and inoculated with rhizobial isolate ARC307 or with gamma rays treated isolates namely; ARC1, ARC2, ARC3, ARC4, ARC5, ARC6 and ARC7. Nodules number, nitrogen content, nitroginase activity and chlorophyll a and b content parameters were decreased by increasing sea water salinity with all used isolates, while proline accumulation parameter increased. At the same time, ARC2 isolate showed the highest values for these parameters above all isolates including the parental isolate ARC307 at all studied concentrations except for proline accumulation parameter, it was the least. Therefore, ARC2 considered as a promising isolate for salt tolerance. Salinity enhanced the occurrence of particular novel proteins in faba bean plants infected with ARC2 isolate.

Key words: N_2 content, nitrogenase activity, salt stress, *Rhizobium leguminosarum*, *Vicia faba*, proline accumulation, SDS-PAGE

INTRODUCTION

Salinity is one of the major environmental threats to agriculture and affects approximately 7% of the world's total land area (Ben-Salah *et al*., 2011) and nearly 40% of the world land surface can be categorized as suffering from potential salinity problem (Payakapong *et al*., 2006). High salinity leads to a decrease in plant and leaf growth and onset of leaf senescence in most crop plants and therefore, to a reduction in total photosynthetic capacity. These effects limit the ability to generate further biomass or to maintain defense mechanisms (Zheng *et al*., 2009). The negative effect of salinity on plant growth has been also attributed to physiological parameters, such as the inhibition of enzyme activities; particularly those involved in the defense against oxidative stress (Turkan and Demiral, 2009). Legumes have been suggested as appropriate crops for the enhancement of bioproductivity and the reclamation of marginal lands, because these plants not only yield nutritious fodder, protein-rich seeds but they also enrich soil nitrogen in symbiotic association with *Rhizobium* (Zaied *et al*., 2005). Nodulation and nitrogen fixation in legume-*Rhizobium* associations are adversely affected by salinity which can preclude legume establishment and growth, or reduce crop yield (Luis *et al*., 2003). Unsuccessful symbiosis under salt-stress may be due to failure in the infection process because of the effect of salinity on the establishment of rhizobia. Legumes and the process of nodule initiation are both more sensitive to salt stress than are rhizobia. The effects of salt stress on nitrogen fixation have been examined by El-Nady and Belal (2005), Hassan (2009) and Kenenil *et al*. (2010). The strategies employed in the last few years to reduce the effect of salt stress on legume production have been focused on a selection of host genotypes that are tolerant to high salt conditions (Kucuk and Kivanc, 2008). Thus, an increase of tolerance to salinity of rhizobial bacteria might constitute another approach to improve plant productivity under symbiosis (Kenenil *et al*., 2010).

Vicia faba (faba bean) is often grown on saline soils in the Middle East and Mediterranean regions. Hassan (2009) showed that sea water affected nodulation of *Vicia faba* and concluded that there may also be effects on infection. Several investigators have demonstrated that amino acid metabolism is strongly influenced by changes in the salinity concentrations (Li *et al*., 2010). In particular, different amino acids are accumulated at different rates under a salt-stressed condition; for example, proline which forms a minor component of the pool of free amino acids in glycophytes, accumulates under stress conditions (Khedr *et al*., 2003). Both chlorophyll fluorescence and hyperspectral reflectance at leaf scale can provide useful tools for non-destructive estimates of plant photosynthetic function under various saltconditions (Li *et al*., 2010). One approach for gaining

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a better understanding of the mechanisms by which plants can respond to salt stress is to study those proteins that are specifically accumulated after exposure of the plants to salinity (Parida *et al*., 2004). The resolving power of one-dimensional polyacrylamide gel electrophoresis allows the detection of minor differences between protein patterns. This has been exploited in a number of studies that have been conducted. The aim of the present study was to assess the effect of salt stress of sea water irrigation on faba bean *Rhizobium* symbiosis relationship through cholorophyll content, nodules number, N_2 content, nitrogenase activity, proline accumulation and faba bean protein profile.

MATERIALS AND METHODS

Materials: *Rhizobium* isolates that used in this study were *Rhizobium leguminosarum biovar viciae* ARC 307 and its gamma rays mutants namely; ARC1, ARC2, ARC 3, ARC4, ARC5, ARC6 and ARC7. These isolates were obtained by treatment with 40 k rad of gamma rays according to Eissa *et al*. (2009). The wild type strain was irradiated in the National Center for Radiation Research and Technology of Atomic Energy Authority of Egypt using Indian gamma cell dose rate 1.0876 K Gy h^{-1} Co^{60}.

Methods: Pots experiment was performed at winter season of 2009/2010 in Department of Genetics, Faculty of Agriculture, Minufiya University, Egypt, according to Ltaief *et al*. (2007). Soil was sterilized by autoclaving at 121°C for 20 min. three times. Autoclaving was repeated after one day to insure complete sterilization and pots were filled with sterilized soil. The moisture level was adjusted to 50% of Water Holding Capacity (WHC) with different concentrations of 0, 20, 25, 30, 40 and 50% of sea water. Faba bean seeds of commercial cultivar Nubaria 1 were pre-germinated to give about 5 mm radicals and inoculated by soaking in different isolate solutions. They were then planted aseptically in the pots. The pots were left at room temperature for eight weeks. Three replicates of each sea water concentration/isolate were conducted then, the plants were taken from pots by removing soil from each pot. Roots were washed in running water and plants were used for further analysis; number of nodules, nitrogen content, nitrogenase activity, chlorophyll content, proline content and protein pattern. Some measurements were conducted in Department of Biotechnology, Faculty of Sciences, Taif University, Kingdom of Saudi Arabia.

Number of nodules: Number of nodules for each isolate at different sea water concentrations was counted. Means at different sea water concentrations of all isolates were calculated.

Nitrogen content: Nitrogen content of plant shoots was determined using the semi-microkjeldahl method. Samples of 0.2 g dry shoots were digested by sulfuric acid (H_2SO_4). Distillation was carried out with 40% NaOH and ammonia was received in 4% boric acid solution. The distillates were then titrated with 0.02 HCl using the mixed methyl red-bromocresol green indicator. Nitrogen percentage was calculated on dry weight basis according to the methods of El-Nady and Belal (2005).

Nitrogenase activity: Nitrogenase activity was determined as C_2H_2 reduction (Serrano and Chamber, 1990). Excised roots from four plants for each isolate at different sea water concentrations were incubated in flasks of 50 mL containing 10% C_2H_2 for 15 min at 28°C. Two samples of 0.5 mL were injected in a gas chromatograph GL Shimadzu (Tokio, Japan) equipped with a Poropack column and a flame ionization detector for the determination of ethylene produced. Nitrogenase activity was calculated from the integration of the chromatograph peaks with regard to a C_2H_4 standard curve.

Chlorophyll determination: Chlorophyll a and b were extracted and measured according to Bidel *et al*. (2007). One plant per replicate for each isolate at different sea water concentrations was used for chlorophyll determination. Prior to extraction, fresh leaves were cleaned with demonized water to remove any surface contamination. Chlorophyll extraction was carried out on fresh fully expanded leaf material; 1 g leaf sample was ground in 2 mL of 85% acetone using a pestle and mortar. The absorbance was measured with a UV/Visible spectrophotometer and chlorophyll concentrations were calculated using the following equations:

$$\text{Chl. a} = 9.784 \times O.D_{662} - 0.99 \times O.D_{644}$$

$$\text{Chl. b} = 21.426 \times O.D_{644} - 4.65 \times O.D_{662}$$

where, O.D, is optical density at the indicated wave length. Chlorophyll content was calculated as mg 100^{-1} g fresh weight of faba bean leaves.

Proline determination: Proline was determined according to the method described by Khedr *et al*. (2003) for each isolate at different sea water concentrations. Approximately 0.5 g of fresh leaf material was homogenized in 10 mL of 3% aqueous sulfo-salicyclic

acid, filtered through Whatman's No. 2 filter paper and 2 mL of solution was mixed with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 mL toluene and cooled to room temperature. The absorbance was measured at 520 nm with a Shimadzu UV 1601 Spectrophotometer. Appropriate proline standards were included for calculation of proline in the sample.

Faba bean protein pattern: The discontinuous buffer system of the high resolution one-dimensional SDS-PAGE technique was carried out for ARC307 and ARC2 isolate at different sea water concentrations (Parida *et al*., 2004). Determination of electrophoretic protein bands in faba bean samples were subjected to protein analysis according to their molecular weights as described by Eissa *et al*. (2009).

Statistical analysis: The data of nitrogenase activity and proline accumulation were analyzed using ANOVAs and subsequent comparison of means was performed using the Duncan test at 5% probability. Multiple regression values were computed with Costat software.

RESULTS

In this study *Rhizobium leguminosarum biovar viciae* ARC307 isolate was used as parental isolate. Also, seven mutants were generated with 40 k rad of gamma rays. All isolates were subjected to inoculate *Vicia faba* plants. Different measurements associated nitrogen fixations were conducted. These measurements included; number of nodules, nitrogen content, nitroginase activity, Chlorophyll a and b content and proline accumulation. Finally, SDS-PAGE protein analysis was carried out for plant shoots inoculated with isolate ARC307 and isolate ARC2 under different sea water concentrations.

Number of nodules: Data in Fig. 1 presented the average number of nodules under various sea water concentrations with different isolates; ARC307, mean of total isolates and ARC2 isolate alone. ARC2 isolate showed the highest values for number of nodules above all isolates including the parental isolate ARC307 at all studied concentrations, therefore, the ARC2 isolate was separately presented because of its promising data. Plants could not form nodules at 30% concentration of sea water with all rhizobial isolates except ARC 2 which had given about 3 nodule/plant. On the other hand, plants could not form any nodule at concentrations 40% and 50% of sea water. The average number of nodules for

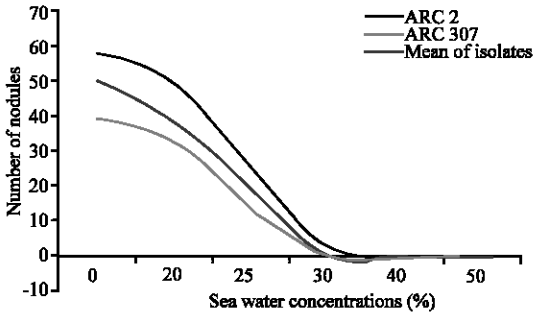

Fig. 1: Histogram for the average number of nodules in *Vicia faba* inoculated with different rhizobial isolates at different sea water concentrations

Table 1: Effect of salt stress on nitrogenase activity of ARC307 isolate and its gamma treated isolates in faba bean

	Nitrogenase activity (µ mole C_2H_4/dry weight nodules)					
Strains	0	20	25	3	40	50
ARC307	15.9^{d}	13.4^{e}	8.6^{h}	NF	NF	NF
ARC1	8.2^{h}	NE	NE	NF	NF	NF
ARC2	18.2^{b}	19.5^{a}	13.5^{e}	6.2^{i}	NF	NF
ARC3	11.5^{f}	6.2^{i}	NE	NF	NF	NF
ARC4	9.8^{g}	3.2^{j}	NE	NF	NF	NF
ARC5	6.4^{i}	NE	NE	NF	NF	NF
ARC6	11.3^{f}	9.2^{g}	8.2^{h}	NF	NF	NF
ARC7	16.4^{c}	11.2^{f}	9.1^{g}	NF	NF	NF

Means followed by the same letter is not significantly different at the p = 0.05 level according to the least significant difference test. NE: Not effective nodule, NF: Not found nodules

ARC2 at 20% concentration was 49.5 nodule/plant while, ARC307 was 32.5 nodule/plant and the mean of the mutated isolates was 38.2 nodule/plant. Moreover, average nodules number at concentration 25% was 24.5, 13.5 and 19.2 nodule/plant for ARC2, ARC307 and mean of the other isolate, respectively. Generally, ARC2 isolate was the best at different sea water concentrations in forming nodules (Fig. 1). In addition, it can form nodules at 30% concentration, while parental and other isolates could not.

N_2 content: Total nitrogen content accumulated in faba bean shoots is presented in Fig. 2. Generally, N_2 content decreased with the increase of sea water concentrations in all isolates. Also, faba bean plants inoculated with ARC2 had showed more nitrogen content above all isolates including the parental isolate ARC307 at all sea water concentrations. On the other hand, only ARC2 and ARC7 isolates survived at the high concentration of sea water (50%). However, ARC2 showed the highest number of N_2 content at all sea water concentrations.

Nitrogenase activity: Data in Table 1 showed nitrogenase activity of faba bean inoculated with different *Rhizobium* isolates at different sea water concentrations. ARC2

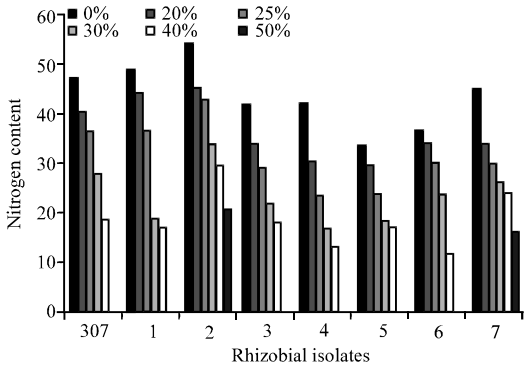

Fig. 2: Histogram for the effect of sea water concentrations on nitrogen content in *Vicia faba* inoculated with different rhizobial isolates

Table 2: Effect of salt stress on proline accumulation of ARC307 isolate and its gamma treated isolates in faba bean

	Proline accumulation (µ mole/g)					
Strains	0%	20%	25%	30%	40%	50%
ARC307	87^{e}	282^{d}	390^{e}	497^{g}	661^{e}	810^{f}
ARC1	84^{f}	283^{d}	436^{b}	596^{b}	672^{d}	850^{b}
ARC2	80^{g}	282^{d}	373^{g}	463^{h}	658^{e}	790^{h}
ARC3	83.7^{f}	286^{c}	423^{c}	578^{d}	706^{c}	836^{d}
ARC4	93^{d}	287^{c}	385^{f}	573^{e}	703^{c}	843^{c}
ARC5	115^{b}	296^{a}	412^{d}	692^{a}	731^{b}	822^{e}
ARC6	118^{a}	292^{b}	472^{a}	508^{f}	747^{a}	857^{a}
ARC7	98.5^{c}	279^{e}	392^{e}	583^{c}	620^{f}	804^{g}

Means within column followed by the same letter is not significantly different at the p = 0.05 level according to the least significant difference test

isolate showed the highest values for nitroginase activity above all isolates including the parental isolate ARC307 at all studied concentrations. In general, nitrogenase activity decreased significantly with increase of sea water concentrations with all isolates except with ARC2 where nitroginase activity increased at 20% sea water concentration and then it decreased. Rhizobium isolates ARC1 and ARC5 showed no nitroginase activity under 20 and 25% sea water concentrations in spite of presence of nodules (Fig. 1). The rest of the isolates showed nitrogenase activity as long as there was nodule formation. Nitrogenase activity was inhibited completely at concentrations 30, 40 and 50% of sea water, while, ARC2 was the only isolate gave nitrogenase activity (about 6.2 µmole) at 30% sea water concentration.

Chlorophyll content: Chlorophyll accumulations through vegetative growth are presented in Fig. 3 and 4. Generally, data showed that chlorophyll a and b content decreased with increase of sea water concentrations with all isolates. However, ARC2 isolate showed the highest values for chlorophyll b content above all isolates including the parental isolate ARC307 at all studied concentrations. ARC3, ARC4 and ARC7 were more sensitive for

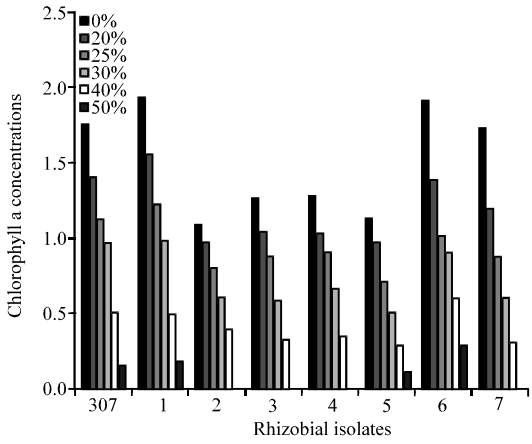

Fig. 3: Histogram for the effect of sea water concentrations on chlorophyll a in *Vicia faba* inoculated with different rhizobial isolates

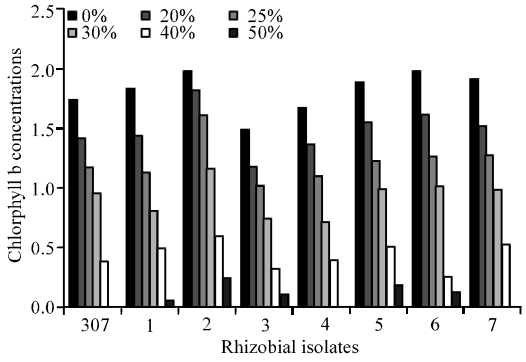

Fig. 4: Histogram for the effect of sea water concentrations on chlorophyll b in Vicia faba inoculated with different rhizobial isolates

chlorophyll a formation to treatment with sea water where it showed no chlorophyll a at 50% sea water concentrations.

Proline accumulation: Data in Table 2 showed proline accumulation of faba bean inoculated with different *Rhizobium* isolates at different sea water concentrations. Generally, proline accumulation increased in response to sea water concentration increment. The highest proline accumulation was found in plants inoculated with AR6 and ARC5 while the lowest was with isolates ARC2. Relatively, ARC2 isolate showed the lowest values for proline accumulation including the parental isolate ARC307 at all studied concentrations.

Protein pattern: Protein pattern of *Vicia faba* plants inoculated with ARC307 or with the mutant ARC2 under different sea water concentrations presented in Fig. 5.

Table 3: SDS-PAGE protein for soluble protein extracted from *Vicia faba* plants inoculated with Rhizobium parental isolate ARC307 or salt tolerant isolate ARC2 at different sea water concentrations

	Sea water concentrations (%)											
	0		20		25		30		40		50	
Molecular weight (kDa)	ARC307	ARC2	ARC307	ARC2	ARC307	ARC2	ARC307	ARC2	ARC307	ARC2	ARC307	ARC2
116	+	+	+	+	+	-	+	+	+	+	+	+
103	-	-	-	-	-	-	-	+	-	-	-	-
97	+	+	-	-	-	-	+	+	+	+	+	+
66	+	+	-	-	-	-	+	+	+	+	+	+
45	+	+	+	+	+	+	-	-	-	-	-	+
40	-	-	-	-	-	-	-	-	-	-	-	+
36	+	+	+	+	+	+	+	+	+	+	+	+
33	-	-	-	-	-	-	-	-	+	-	-	-
29	+	+	+	+	+	+	+	+	+	+	+	+
27	-	-	-	-	-	-	-	+	-	-	-	+
24	+	+	+	+	+	+	+	+	+	+	+	+
20	-	-	-	-	-	-	-	-	-	-	-	+
14	+	+	-	-	-	-	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+

(+): Presence, (-): Absence

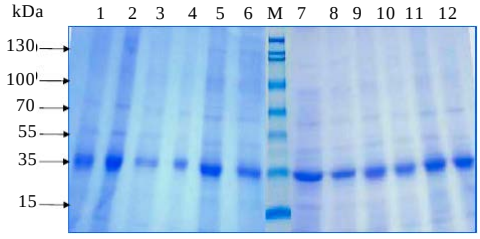

Fig. 5: Effect of salt stress on SDS-PAGE protein pattern of faba bean inoculated with parental isolate ARS 307 or with its tolerant salt isolate (ARC2) at different sea water concentrations. (1) ARC3 with 0% sea water (2) ARC2 with 0% sea water (3) ARC3 with 20% sea water (4) ARC2 with 20% sea water (5) ARC307 with 25% sea water (6) ARC2 with 25% sea water (7) ARC307 with 30% sea water (8) ARC2 with 30% sea water (9) ARC307 with ARC307 with 40% sea water (10) ARC2 with 40% sea water (11) ARC307 with 50% sea water (12) ARC2 with 50% sea water (M) molecular weight maker

Table 3 showed the banding pattern of each treatment and their molecular weight different. Four bands with molecular weight 36, 29, 24 and 6 kDa were present in all sea water concentrations with faba bean inoculated with rhizobial isolate ARC307 and its treated isolates which, it could be considered as common bands, while other ten bands were polymorphic. There were present unique bands of molecular weight 103 and 20 kDa. that were present in plans inoculated with ARC2 under 30 and 50% sea water concentrations, respectively. At the same time, the band with molecular weight 116 kDa. was uniquely absent with ARC307 isolate at 0% sea water concentration.

DISCUSSION

In this study, a fundamental question was addressed about the evaluation of the isolate ARC307 and its mutants on nitrogen fixation efficiency under sea water stress. Many parameters were conducted which includes; nodule formation, nitrogen content, nitroginase activity, chlorophyll a and b formation and proline accumulation. Finally, SDS-PAGE protein pattern was generated for both the parental isolate and the best evaluated isolate (ARC2).

The results showed that increasing sea water concentrations decreased the average number of nodules. Thus, nodule formation was sensitive to sea water stress under this experiment conditions which agreed with Tejera *et al*. (2004). However, ARC2 isolate was more tolerant to sea water stress than the rest of the isolates including the parental isolate 307. At the same time, it was the only isolate formed nodules at 30% sea water concentration. Sea water inhibits nodule formation by the inhibition of initial steps of Rhizobium-legume symbioses. Parallely, nitrogen content reduction increased with sea water concentration increment for all isolates. However, isolate ARC2 showed the least reduction. Reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules (Kenenil *et al*., 2010). The depressive effect of salt stress on N_2 fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (Zahran, 2001) who found that the nitrogen content in soybean was more strongly reduced with increased in irrigated salinity water. Also, the salt-induced distortions in nodule structure could be the reason for the decline in the N_2 fixation rate by legumes subjected to salt stress.

As for the data of nitroginase activity of formed nodules, the isolates under study can be divided into three categories based on nodules efficiency under sea water concentrations 20 and 25%. Higher sea water concentration was excluded since there was no nodule formation except for ARC2. First category includes tolerant isolates; parental isolate ARC307, ARC2 and ARC 6 and ARC7. Second category includes moderately tolerant isolates ARC3 and ARC4. Finally, sensitive isolates ARC1 and ARC5. This result was in agreement of data of Ashwani and Vandna (2003) who noticed strong inhibition in nitroginase activity during growth in salinity conditions.

For Chlorophyll a and b, plants inoculated with different isolates showed reduction, while plants inoculated with ARC2 showed the least reduction. This results agreed with Li *et al.* (2010) who noticed reduction in photosynthetic activity under salt stress Chinese castor bean at increasing levels of salt stress.

Proline content in plants inoculated with different isolates showed increment with increasing sea water stress, while plants inoculated with ARC2 showed the least proline levels. Many legumes adapt to saline conditions by the intracellular accumulation of low-molecular-weight organic solutes called osmolytes (Hassan, 2009). The accumulation of osmolytes is thought to counteract the dehydration effect of low water activity in the soil. The concentrations of proline in the plant tissues were generally very low but the levels were significantly affected by salinity, (Jampeetong and Hans, 2009). Also, they found that concentrations of proline were about three times higher in the plants grown at 150 mM salinity compared to the control. Salt stress results in the formation of specific proteins in legumes (Parida *et al*., 2004), reported that the production of 41 proteins was increased at least 10-fold in salt-stressed. Proline has been suggested to play multiple roles in plant stress tolerance. It acts as a mediator of osmotic adjustment, protects macromolecules during dehydration and serves as a hydroxyl radical scavenger.

For protein pattern study, two unique bands of molecular weight 103 and 20 kDa were appeared in plants inoculated with the tolerant ARC2 isolate. Zahran *et al.* (1994) reported the appearance of new protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of rhizobia from woody legumes grown under salt stress. Garcia *et al.* (2005) obtained protein bands with molecular weight 42 and 45 kDa on SDS-PAGE related to stress. Salinity affected shoots growth more than root growth, as was also reported by Chourey *et al.* (2003) who demonstrated that salinity stress was able to trigger the accumulation of several major stress proteins. They also stated that the accumulation levels of these proteins correlated with stress tolerance in the various plant species, suggesting protective roles under osmotic stress and that recovery from salt stress was consistently accompanied by degradation of the salt-stress induced proteins. Parida *et al.* (2004) reported that SDS-PAGE analysis showed nearly identical protein profiles in control and salt treated samples which suggest that salt did not alter protein synthesis or proteolytic activity. The decrease in soluble protein of the nodules may be due to a protein break-down or to an alteration in the incorporation of amino acids into proteins.

In conclusion, faba bean plants inoculated with ARC2 isolate showed higher values in number of nodules, nitrogen content, nitroginase activity, chlorophyll a and b content but there were the least in proline accumulation. ARC2 might be considered as salt tolerant isolate under our experimental conditions. Also, the plants inoculated with this ARC2 showed new protein bands. Further analyses of this mutant and its genetic construction are needed to for more understanding of its tolerant ability to salt stress.

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