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Effects of *Rhizobium* Strain on the Growth, Nodulation, N_2 Fixation and Ions Accumulation in *Vicia Faba* Plant Under Salt Stress

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ABSTRACT

Vicia faba L.-rhizobia symbiosis is utilized in different biological ways to improve the productivity of faba beans. This research aims to analyze the effects of the *Rhizobium* strain on nodulation, N_2 fixation, growth and ion accumulation under salt stress in *Vicia faba*. The commercial cultivar of faba beans (*Vicia faba* L. var. minor) was inoculated with the *Rhizobium leguminosarumbiovar,* by considering *viciae* strains S10 and S16, after 15 days of growth. This inoculation was carried out in the solution culture consisting of two salt concentrations; 0 mmole l^{-1}NaCl and 50 mmole l^{-1} NaCl. The results revealed that under saline and non-saline conditions, S10 and S16 strains of *Rhizobium leguminosarum* resulted in the formation of ineffective and effective symbiosis with faba beans. However, the presence of salt stress resulted in increasing the biomass of nodule and nitrogen content. The concentrations of sodium and chloride, in shoot and root, were increased in the presence of salinity. However, potassium concentration was only increased in the shoot. With and without salinity, phosphorus concentration in the shoots was not modified. The results revealed that the salt tolerance of faba beans, inoculated with two strains of *Rhizobium* were found to possess association with their stable growth. Moreover, the salt tolerance of faba beans inoculated with two salts tolerant rhizobia was also associated with an increment in the capacity of faba beans to increase nodulation and the concentration of shoot N_2, Na and Cl^- content. In addition, salt tolerance of this variety, inoculated with *Rhizobium* strains was associated with a decrement in the concentration of K^+ in shoot under the salt constraints.

Key words: Ions accumulation, N_2 fixation, *Rhizobium* strain, Salinity, *Vicia faba*.

INTRODUCTION

Legume is recognized as the most beneficial plant because of having high grain protein contents and ability to fix biological nitrogen (Khan, 2018). Soil salinity is a major constraint that limits the agricultural productivity of Legumes (Morgan *et al.* 2018; Dutta and Bera, 2014). Basically, salt stress has a negative influence on the production of legume crop. The impacts of salt stress are further multiplied in the cases when plants get N_2 from the symbiotic fixation (Singleton and Bohlool, 1984). Salinity tolerance varies among different crops of legumes, which could be understood by considering the assertions of different researchers. According to Hansen and Munns (1985), *Sesbania Grandiflora* has halophytic adaptation mode, in the presence of salinity. Moreover, Rhodes and Felker (1988) stated that *Prosopis* and *Acacia* have a high tolerance against salinity. Contrarily, Lauchli (1984) stated that the grain legumes are moderately tolerant or sensitive to salinity. Elsheikh and Wood (1990) also affirmed that *Vigna radiata* and *Cicer arietinum* are highly sensitive to salinity.

The symbiotic fixation of N_2 within a legume genotype is strongly affected by partner strains of *Rhizobium*. Udvardi and Day (1997) affirmed that the process of N_2 fixation includes complex metabolic interfaces between *Rhizobium* bacteroids and cytoplasm of the infected cells of the host plant. Singleton *et al.* (1982) stated that as compared to the respective host regimes, the growth and survival of *Rhizobium* demonstrate high tolerance in vitro under the influence of high osmotic pressure. However, according to Mohammad *et al.* (1989), the strains and species of *Rhizobium* possess different properties, which further influence their tolerance levels for different salts.

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Salinity can be understood as abiotic stress that affects the productivity of the plants. Under the influence of osmotic effects, salinity result in stomatal closure, inhibit CO_2 fixation and disturb CO_2/O_2 ratio in leaves. Hu *et al.* (2005) stated that detrimental effects in plants are caused by salt concentration that is accumulated in the tissues of the plants; however, salinity-induced growth reduction in plants is not resulted due to toxicity caused by Na^+ and Cl^-. Chen *et al.* (2005) found that the efficacy of salt treatment for depleting cellular K^+pools and stimulating K^+ efflux is interlinked with the sensitivity of cultivar to salinity. Borsani *et al.* (2001) stated that in tomato and *Arabidopsis* mutants, hypersensitivity negatively impacts the ability of the plant to absorb K^+. Attia *et al.* (2008) demonstrated that $Ca^{2+}$and K^+ limit the growth of *Arabidopsis,* specifically when these two ions and NaCl are present in the roots; however, there is a different situation when the roots of the plants are kept in the control medium. The potential impacts of a combination of legume-*Rhizobium* strain on symbiotic Nitrogen fixation, under salt stress, are required to be further examined. Moreover, ion accumulation levels and plant's activities, as tracers, are also required to be further investigated (Waisel, 1989). This work was conducted for investigating the effect of *Rhizobium* strains on growth, nodulation, N_2 fixation and ion accumulation in the faba bean plants under salt-stress.

MATERIALS AND METHODS

Plants material and culture conditions

The faba (Bachar) bean have been widely utilized for agricultural purpose in Tunisia. Seeds isolated from soil in two sites of Tunisia, were inoculated separately with either S10 or S16, which are strains of salt-tolerant *Rhizobium*. The seeds were grown in the controlled environment and under the greenhouse conditions with the plants grown in pots. The seeds were sterilized by using calcium hypochlorite (2%) and bathed by using sterile distilled water. Afterwards, the seeds were germinated in soft agar comprising Bergersen solution (100 mL) at 28°C (Vincent, 1970). The *Rhizobium legumino sarum* biovar was used for preparing the rhizobia inoculant. The salt-tolerant *Rhizobium* strains (S10 and S16) were preserved in tubes at the temperature of four-degree centigrade YEM media. Rhizobia were grown into the solution of liquid YEM, presented within Erlenmeyer flask, at 28°C, in darkness. The 4-day-old seedlings were soaked into inoculants (100 mL) comprising approximately 10^8 cells m/L for 30 minutes duration. After inoculation, the seedlings were placed into the plastic pots, comprising of 1 Kg sterilized perlite. The seedlings were then categorized into two groups (plants per treatment). Each plant was moistened with the nutrient solution comprising of 50 mM NaCl (intervention group) and 0 mM NaCl (control group). After 45 days of culture, corresponding to the onset of flowering, plants were separated and the dry matter of shoots, nodules, and roots were evaluated.

Determination of P and N contents in roots and shoots

Molybdate blue method, suggested by Murphy and Riley (1962) was utilized to determine the N and P contents in roots and shoots. Approximately 0.3 g of dry weight was thermally treated (undergo incineration) and filtered by using Whatman filter paper. Moreover, distilled water was added to acquire 50 mL volume. Within 2.5 ml mixture, comprising of hydrazine sulfate (0.15%) and ammonium molybdate (2.5%), 100 µl of each extract was further added. The mixture was incubated for 30 minutes at room temperature and absorbance was read at 820 nm. The Kjeldahl method was used for determining N_2 content, by considering shoot and root samples (0.5 g).

Determination of K^+, Cl^- and Na^+, contents in roots and shoots

Distilled water was added in 0.3 g of dry mass (incinerated and filtrated through Whatman filter paper), for getting 50 mL final volume. The concentration of Na^+ and K^+ was examined by spectrophotometry (Jenway flame types), whereas, Cl^- concentration was analyzed by using coulometry (Butcher Cotlovechloridometer).

Statistical analysis

Plant harvesting was performed at the early pod-stage of flowering (45 days after seeding). Shoots, roots, and nodule components of plants were also separated. These components were dried for the duration of 2 days at 70°C. Ion accumulation was investigated by weighing each fraction. All traits were determined by considering the standard deviation of 0.05.

RESULTS AND DISCUSSION

Biomass and number of nodule

When the faba beans were inoculated with S16 and S10, variation in a number of nodule and nodule growth was observed (Fig 1A and 1B). In the absence of salt, the plants that were inoculated with S10 and S16 indicated 8.5 and 7.75 nodules per plants, respectively. Moreover, the plants that were inoculated with S10 and S16 were found to possess nodule biomass of 0.052 g DMs/plant and 0.025 g DMs/plant respectively. When the impacts of inoculation of the considered strains were analyzed, it was revealed that salinity resulted in increasing the number of nodules and also facilitated nodule growth in faba bean plants (Fig 1A and 1B). In addition, the nodule dry weight was also increased up to 30% and 90%, under the action of S10 and S16 strains. Moreover, it has also been recognized from the results that nodules are more tolerant to salinity, with 50 mM NaCl as compared to 0 mM NaCl.

Fig 1B indicates that salinity had positive impacts on the total nodule number. This aspect indicates that *Rhizobium* strains S10 and S16 were tolerant to medium levels of salinity (50 mmoles/l). These outcomes contradict the results of the experiment that was conducted for chickpea

plants, in which 50 mmoles/l of NaCl decreased nodulation by 60 % (Elsheikh and Wood, 1990). However, it was found that after the period of 4 weeks, nodulation in chickpea plant that was receiving the highest saline treatment was completely depressed. For *Vigna radiata*, the exposure to plants to low salinity levels (> 50 mmoles/ l) reduced nodulation to 50 % (Hafeez *et al*, 1988).

This experiment reported increment in the average weight of nodule under salinity that validates the observations, taken for faba beans and chickpeas. However, the study of Zahran and Sprent (1986) did not report any increment in the weight of nodule. Minimization in the formation of the nodule at low salinity levels could be because of the adverse influences of salinity on nodule initiation process. A nodule initiation, occurred during *Rhizobium*-legume symbiosis has high sensitivity to the osmotic stress. Salinity tolerance in *Bradyrhizobium* and *Rhizobium* is higher than the legume host that reveals that the successful symbiosis is dependent on the tolerance levels of the host plants (Bhardwaj,1975).

Shoots and roots biomass

In the control group, faba bean shown different growth patterns for roots and shoots when they were inoculated with different rhizobia strains (Fig 1C and 1D). More productivity levels were observed in the plants that were inoculated with S10 as compared to S16. The shoot biomass was 0.8 g DMs/plant for the plants that were inoculated with S10 and 0.675 g DMs/plant, in case of inoculation with S16. It was observed that the impacts of salinity on growth of shoots and roots varied between the plants that were inoculated with both strains, such that inoculation with S10 resulted in decreasing less weight, as compared to S16 (Fig C).

The evidence reported that salinity reduces the weight of roots and shoots in several legumes, including green bean (*Phaseolus Vulgaris*) (Gulmezoglu, 2016), Soybean (*Glycinemax*) (Grattan and Maas, 1988), and faba bean (Yousef and Sprent, 1983). It has also been reported that in 90 mmoles/l NaCl, the cowpea plants (*Vigna radiata*) survived till 65 days (maturity) (Hafeez *et al.*, 1988); whereas, chickpea plants (*Cicer arietinum*) survived only for the duration of 30 days (Elsheikh and Wood, 1990). In this research, the roots and shoots of faba beans that were inoculated with different strains of salt tolerant rhizobia were not affected at 50 mM NaCl (Fig 1C and 1D).

Nitrogen content

Fig 2B and 2D presented that in the presence of salt, the faba beans that were inoculated with S10 and S16, accumulated more concentration of N_2 in shoots, as compared to the roots. On the contrary, without salt, N_2 concentration was less in shoots, as compared to the roots. Salinity increased the concentration of N2 in roots and shoots of plants that were inoculated with S16. However, no such behavior was observed in the roots and shoots of

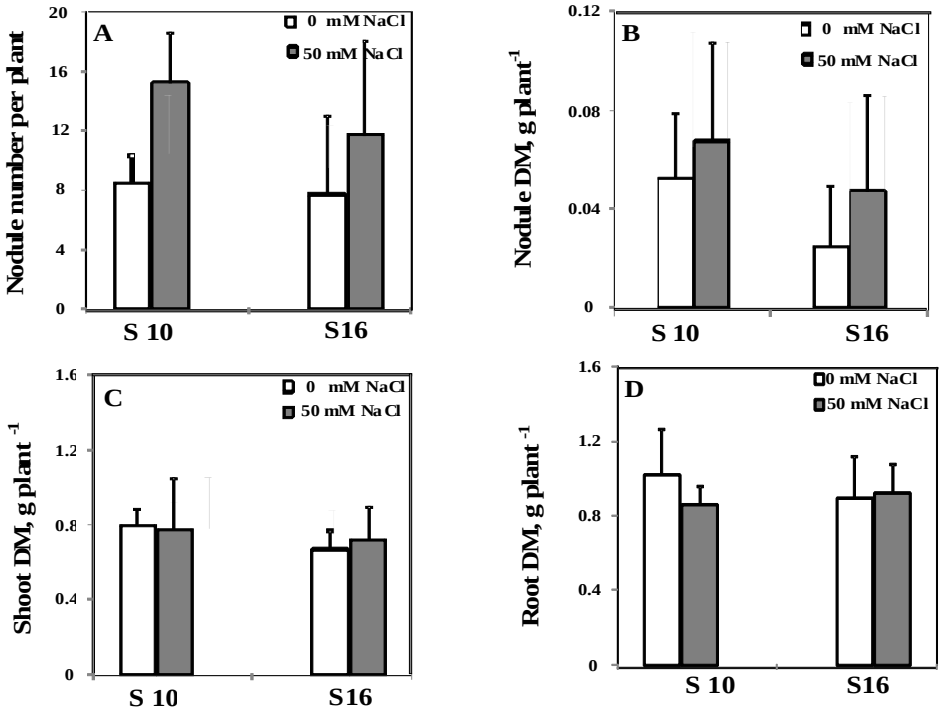

Fig 1: Effect of NaCl (50 mM) on nodule number (A) and nodule (B) shoots (C) and roots (D) biomass of faba bean variety: Bachar. NaCl 50 mM was added after 15 days of growth. Plants were harvested 45 DAS (the day after sowing). Data are the means ±SD of four replicates.

the plants that were inoculated with S10. The faba bean plants presented the opposite behaviour for N_2, as compared to K^+.

Demonstrating significant increment in biomass of nodule and the number of nodules, the research outcomes indicated that growth of faba beans inoculated in S10 and S16, under the saline condition, resulted in the accumulation of more concentration of N_2 in shoots (Fig 2B). These results validated the findings of Weil and Khalil (1986) and Singleton (1983) by representing no reduction in the N_2 concentration.

The increment in biomass and number of nodules in faba bean, due to salinity, can be considered as a systematic response of faba beans to fulfil high N_2 demands that are required for plant growth under salt treatment. The effectiveness of rhizobial symbiosis for faba beans, under salinity, was significantly increased; thereby, indicating that salinity altered the respiratory cost of N_2 fixation and contribution of N_2 in the plant's growth.

Na^+, Cl^-, K^+ and P accumulation

Fig 3A, 3B, 3D, 3E presented that in the absence of salinity, Cl^- and Na^+ concentrations in shoots and roots was higher within the plants that were inoculated with S16. Salinity also resulted in increasing concentrations of Na^+ and Cl^- in shoots and roots; however, less accumulation of Cl^- was found in the plants that were inoculated with S16. Figs 3C and 3F presented that salinity resulted in declining the concentration of K^+ in the shoot while increasing the concentration of K^+ in roots. Without salt, the shoot accumulated higher K^+ concentration; however, K^+ concentration in roots was higher than shoots. Fig 2A and 2C presented that the accumulation of P within shoots was not modified by the presence and absence of salt; however, the concentration of P was decreased in the shoots of such plants that were inoculated with S16 and increased in shoots of the plants that were inoculated with S10.

Higher concentrations of Cl^- and Na^+ on shoots and roots in such plants that were inoculated with S10 and S16 did not significantly hinder the growth of plants (Fig 3A, 3B, 3D, 3E), as compared to the plants that were not challenged by salinity (Fig 1C, 1D). The culture in hydro-aeroponic configuration is very difficult to be alleviated by the adverse impacts of salt, specifically in the salt tolerant species; thereby, indicating significant impacts of the absorbed Cl^- and Na^+ ions.

Salinity adversely affected K^+ intake (Fig 3C) that validates the research outcomes presented by Ushakova *et al.* (2005). The K^+ intake is strongly influenced by competition between Na^+ and K^+ at absorptionsites for being uptaken through common transport systems (Khan *et al.*, 2000 a-b). The transport systems are highly selective for K^+. This behavior facilitates leakage of Na+and results in a significant reduction of K^+ accumulation, which is a response characteristic to different halophytes. Decrement in the efficiency of plants to uptake K^+ is resulted due to direct competition within root transporters to uptake K^+ and Na^+. K^+ and Na^+ possess similar physiochemical properties;

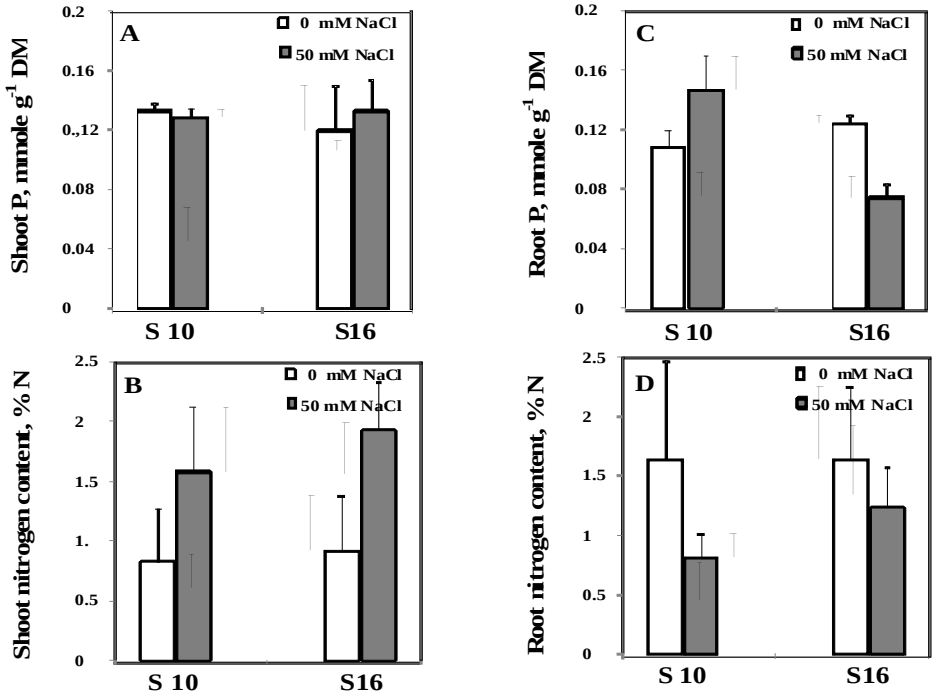

Fig 2: Effect of NaCl (50 mM) on Phosphate (A) and Nitrogen(B) contents in shoots and Phosphate (C) and Nitrogen(D) contents in roots of faba bean variety: Bachar. NaCl 50 mM was added after 15 days of growth. Plants were harvested 45 DAS (the day after sowing). Data are the means ±SD of four replicates.

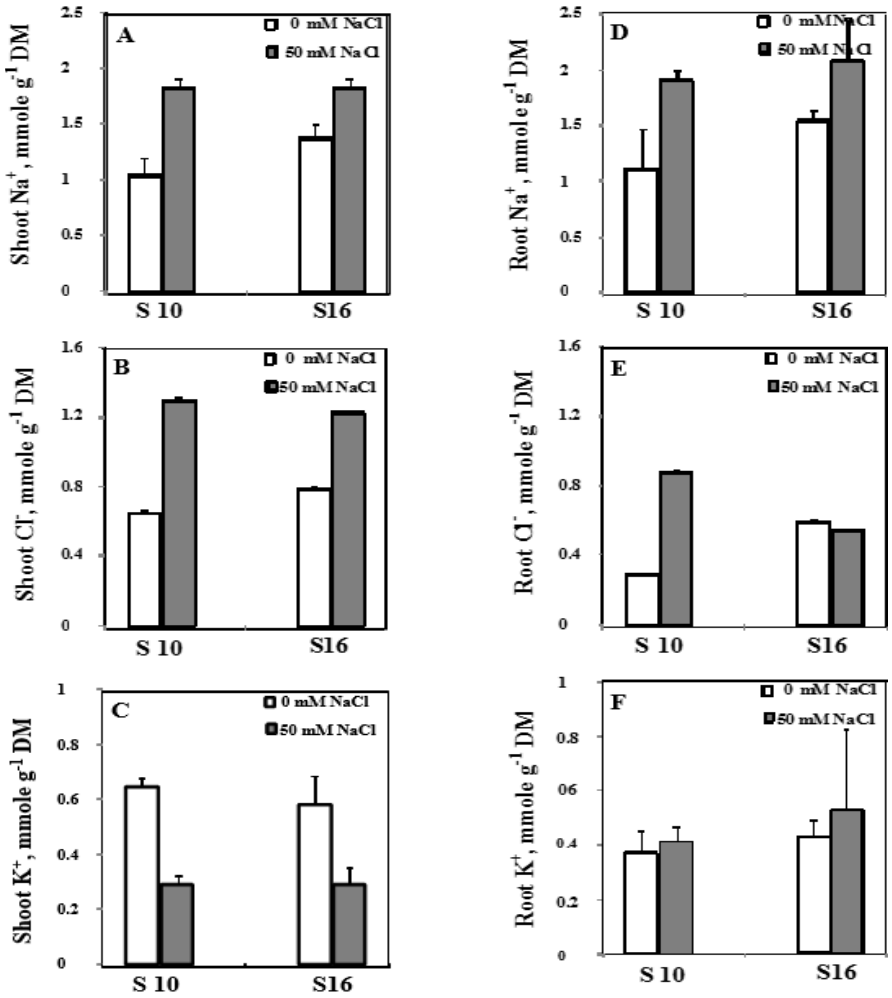

Fig 3: Effect of NaCl (50 mM) on Na^+ (A), Cl^- (B) and K^+ (C) contents in shoots and Na^+ (D), Cl^- (E) and K^+ (F) contents in roots of faba bean variety: Bachar. NaCl 50 mM was added after 15 days of growth. Plants were harvested 45 DAS (the day after sowing). Data are the means ±SD of four replicates.

however, a high concentration of Na^+ strongly inhibit K^+ uptake by roots.

Similar to *Medicago Sativa* (Irigoyen *et al.*, 1992) and *P. vulgaris* (Raggi, 1994), chickpeas also accumulate free proline in nodules and leaves, under the influence of hyperosmotic stress. However, free proline accumulation does not necessarily present tolerance to osmotic constraints. The increased concentration of proline in some of the bean cultivars is often observed due to osmotic stress. The greatest increment in proline was found within the drought-susceptible cultivars. On the contrary, negative association between proline accumulation and salt tolerance was also highlighted in previous researches. The stability in the accumulation of P, specifically in the shoots, might result due to respiratory activity within nodule tissues, for producing malate for osmotic adjustment, similar to guard-cells carrying out osmoregulation by using K^+-malate (Tablott and Zeiger, 1998). K^+, malate and mannitol have been reported to cause a decrement in osmotic potential in numerous species (Peltier *et al.*,1997). In *M. truncatula*, the concentration of Na^+ (1-1.5 mmol/g DW) is two times higher than Cl^-under salinity; therefore, malate might be used for offsetting the anion deficit. However, this condition may resist with the malate bacteroid supply for N_2 fixation; thereby, resulting in inhibiting N_2-dependent growth, irrespective of increment in O_2 consumption in nodules. Phosphoenolpyru--vate (PEP) synthesises nodule malate and PEP carboxylase acts as a catalyst in the synthesis of malate. The malate is stimulated by salt in ILC1919 (salt-tolerant chickpea) that further increases the concentration of nodule malate in roots (Soussi *et al.*, 1999).

CONCLUSION

Salt tolerance in the olive trees is strongly influenced by a reduction in uptake or transport of saline ions. The tolerance to salinity stress in faba beans, inoculated with S10 and S16 was characterized by excluding Na^+ and Cl^- from the aerial part. Moreover, nodulation of faba beans was increased, whereas, the growth of faba beans plants was not significantly affected by 50 mMNaCl.

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