

Effects of *Rhizobium leguminosarum* Inoculation on Growth, Nitrogen Uptake and Mineral Assimilation in *Vicia faba* Plants under Salinity Stress

L. Benidire^{1,2}, M. Lahrouni³, F. El Khalloufi⁴, M. Göttfert², and K. Oufdou^{1*}

ABSTRACT

Salt stress constitutes one of the most significant environmental constraints that limit legume production, especially in arid and semi-arid regions. This study aimed to evaluate the effect of salt stress (0, 60, and 120 mM of NaCl) on growth, nodulation process, nitrogen uptake and mineral nutrition content of *Vicia faba* L. plants inoculated with native Moroccan rhizobia isolated from root nodules of faba bean plants grown in the Marrakech-Haouz region. Three *Rhizobium leguminosarum* strains (RhOF34, RhOF125 and RhOF15), which had different tolerance to salinity, were used to inoculate faba bean plants. The results showed that chronic exposure to salinity affected growth and symbiotic parameters of *V. faba* differently. Shoot biomasses were reduced under salinity stress especially in the plants inoculated with the salt sensitive strain (RhOF15). The nodulation of faba bean roots sharply decreased under 120 mM salt treatment, particularly with the sensitive *Rhizobium* strain. The total nitrogen content decreased with increasing salinity, except for the plants inoculated with the tolerant strain RhOF34, which kept a high nitrogen content. Sodium and calcium concentration increased sharply in plant tissues with increasing salt stress, while the potassium concentration decreased. RhOF34 strain reduced Na⁺, Ca²⁺ and K⁺ absorption by faba bean plants. Inoculation with the salt tolerant strains RhOF125 and RhOF34 led to an increased plant biomass, nodules number, and nitrogen content; and seemed to protect faba bean plants against the toxic effects of salinity.

Keywords: Mineral nutrition, Plant nutrition, Rhizobia, Salt tolerance, Salt tolerant strains.

INTRODUCTION

Soil salinization is one of the major factors limiting crop production, particularly in arid and semi-arid regions (Paranychianakis and Chartzoulakis, 2005; Ahmed, 2009; Shrivastava and Kumar, 2015). Human activities and particularly improper irrigation management of arable land may strongly modify water balance and may cause salt accumulation under limited drainage conditions, thus, accelerating land

degradation in arid and semi-arid environments (Zalidis *et al.*, 2002; Ahmed, 2009). Almost 27.3 million ha of surface land located in the Mediterranean basin is affected by salinity-related problems (Aragüés *et al.*, 2011).

The plant response to salinity depends on many factors such as the stage of plant growth, the growing conditions, the concentration and type of salt. In addition to osmotic effects, the plants are also affected by toxic damages resulting from a stress

¹Laboratory of Biology and Biotechnology of Microorganisms, Faculty of Sciences Semlalia, Cadi Ayyad University, P. O. Box: 2390, Marrakech, Morocco.

* Corresponding author; e-mail: oufdou@uca.ma

²Dresden University of Technology, Institute of Genetics, Helmholtzstr. 10, D-01069 Dresden, Germany.

³Department of Biology, FST Errachidia, Moulay Ismail University, Errachidia, Morocco.

⁴University Hassan 1st. Polydisciplinary, Faculty of Khouribga, BP.145, 25000 Khouribga, Morocco.



specific to Cl^- and Na^+ , which lead to physiological and biochemical changes and, consequently, inhibit plant growth (Heidari and Jamshid, 2010). Salt affects major processes besides growth, photosynthesis, protein synthesis, energy and lipid metabolism (Ramoliya et al., 2004; Parida and Das, 2005; Valizadeh et al., 2013; Oufdou et al., 2014). High level of salt results in nutrient imbalance caused by the loss of control of nutrient uptake and/or transport to the shoot leading to ion deficiencies (Munns, 2002; Heidari and Jamshid, 2010).

Salinity is considered as a threat to the food supply because most crops do not grow under high salt concentrations (Flowers, 2004; Kumari and Mesfin, 2015). Therefore, and in order to increase food production, it seems necessary to determine the potential of crops to tolerate salinity (Athar and Ashraf, 2009). In developing countries, legumes play a key role in sustainable agriculture and present economic and environmental benefits due to their important capacity to fix atmospheric nitrogen in the root nodules in symbiosis with rhizobia. Symbiotic rhizobia can increase yields, accelerate flowering/fruit ripening and contribute to the improvement of the soil nitrogen balance for the benefit of legumes and other species associated with legumes (Brunel et al., 2007; Jia and Gray 2008). Hence, by growing legumes, farmers might be at least partially exempted from the use of costly chemical nitrogen fertilizer.

Variability in salt tolerance among crop legumes has been reported by Zahran (1991). Some legumes, e.g. *Vicia faba*, *Medicago sativa* and *Trifolium alexandrinum*, are more salt tolerant than others such as, e.g., *Vigna*, *Glycine*, and *Phaseolus spp.* (Zahran, 1991; Zahran, 1999). The effect of NaCl salinity on legumes' growth, symbiotic development, and nitrogen fixation has been the subject of several investigations (Elsheikh and Wood, 1990; El-Hamdaoui et al., 2003; Borucki and Sujkowska, 2008; Oufdou et al., 2014). These studies showed that salinity stress

affects negatively the nodulation capacity of legumes and nitrogen fixation. For some rhizobia, the upper limit for salinity tolerance appears to be higher than that of their host legumes (Kassem et al., 1985; Abdelmoumen et al., 1999).

We focused our research on *Vicia faba* L. (faba bean), which occupies about 40% of the total area (approximately 197,000 ha) of legumes planted in Morocco (MAPM, 2012). The aim of the present work was to determine the effect of inoculation of faba bean with native Moroccan rhizobia strains on the growth of the plants, their nodulation, the nitrogen uptake and the mineral nutrition assimilation under saline conditions. Further, the effect of tolerant and sensitive rhizobial strains on faba bean plants under salinity exposure was compared to suggest the most efficient nitrogen fixing symbiont.

MATERIALS AND METHODS

Isolation and Purification of Rhizobial Strains

Rhizobial strains were isolated from root nodules of *V. faba* plants collected from Marrakech Haouz region (Morocco). Nodules were disinfected with sodium hypochlorite (1.2% Cl) and washed several times with sterile physiological water (9 g L^{-1} of NaCl). Nodules were crushed in a sterile tube. The suspension was streaked on Petri dishes containing Yeast Extract Mannitol (YEM) agar medium with Congo red (Vincent, 1970). Plates were incubated at 28°C for 48 to 72 hours, individual rhizobia colonies showing little or no Congo red absorption and characterized by a gluey aspect were further purified by repeated streaking on YEM medium agar. Pure isolates were checked for their nodulation capacity using aseptic *V. faba* seedlings grown in sterile sand, and stored at -25°C in glycerol 30%.

Isolates were firstly checked for their infectivity and were screened for their ability to tolerate high salinity (NaCl) using

YEM agar medium. Inoculated plates were incubated at 28°C and after 2 to 3 days the growth of colonies was monitored visually. A strain was considered tolerant if its growth was similar to that observed on the control plate (0 mM of NaCl, temperature 28°C and pH 7).

During this study, we worked with two osmotolerant rhizobia strains RhOF34 and RhOF125, tolerant up to 7.5 (128.3 mM) and 6 g L⁻¹ (102.7 mM) of NaCl, respectively, and a sensitive strain (RhOF15) which tolerated up to 3.5 g L⁻¹ of NaCl (59.9 mM).

Molecular Characterization of Rhizobial Strains

DNA Extraction

The extraction of genomic DNA was conducted according to the protocol of Dhaese *et al.* (1979). After 48 hours of incubation at 28°C in YEM medium, bacteria from 4 mL of the culture were collected by centrifugation. The bacterial biomass was washed with TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and resuspended in 300 µL TE buffer. Later, 100 µL of 5% SDS and 100 µL pronase E (2.5 mg mL⁻¹ in TE buffer) were added. After mixing, the solution was incubated over-night. The DNA was then mixed with 300 µL of Tris-equilibrated phenol solution. The mixture was centrifuged at 15,000 rpm for 3 minutes. The DNA was further purified with 300 µL of chloroform-isoamyl alcohol (24:1, v/v) and centrifuged at 15,000 rpm for 5 minutes. DNA from the aqueous phase was precipitated with 2.5 volumes of absolute ethanol. The samples were centrifuged for 10 minutes at 14,000 rpm at 4°C. The resulting DNA pellet was washed with 70% ethanol. After centrifugation for 15 minutes at 13,000 rpm at 4°C and removal of the liquid phase, the DNA pellet was vacuum dried, and solubilized in 100 µL of sterile Milli-Q water. The purity and the quantity of

extracted DNA were determined using a NanoDrop.

PCR Amplification of 16S rDNA, Sequencing and Analyses

The 16S rDNA was amplified using primers 16Sa (5'-CGCTGGCGGCAGGCTTAACA-3') and 16Sb (5'-CCAGCCGCAGGTTCCCCT-3') (van Berkum and Fuhrmann, 2000). The reaction mixture with a total volume of 50 µL, was composed of rhizobial DNA (100 ng), Dream Taq buffer, dNTP (100 pmol), Dream Taq polymerase (1.25 U) and sterile Milli-Q water. PCR conditions were: an initial cycle of denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C at 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1.5 minutes; and a final extension at 72°C for 10 minutes. The PCR products were checked by horizontal agarose gel electrophoresis (1% (w/v) agarose in Tris-Acetate-EDTA buffer). The PCR products were purified by "MEGAquick-spin™ Total Fragment DNA Purification Kit". Sequencing was done by GATC Biotech (Konstanz, Germany) using primers 16Sa, 16Sb and two internal primers. For blast searches, the resources of the National Center for Biotechnology Information (Johnson *et al.*, 2008) were used. Phylogenetic analysis was conducted with MEGA version 6 (Tamura *et al.*, 2013).

Biological Material and Growth Conditions

Moroccan faba bean seeds (*V. faba* L. var. Alfia 5) were surface-sterilized with sodium hypochlorite (1.8% Cl) for 15 minutes, followed by rinsing them several times with sterile distilled water. The seeds were pre-germinated for 4 days on wet filter paper in Petri dishes. The pre-germinated seeds were put in pots containing 2 kg of sand (three seedlings in each pot). The sand, used as a neutral substrate, was previously washed



two times with distilled water and dried at ambient temperature for three days and then 5 kg of sand was poured into stainless steel plates (Dimensions: 44×31 cm h: 3 cm). These plates were placed in the muffle furnace at 200°C for 4 hours to sterilize the sand.

The pots were separated into three groups (12 pots per group); each group was inoculated with 1 mL of liquid medium containing 10^9 cells mL⁻¹ of one strain (RhOF34, RhOF125 or RhOF15). Plants were grown in the greenhouse of the Faculty of Sciences Semlalia (Marrakech, Morocco) under natural conditions and irrigated three times a week with Rigaud and Puppo nutrient solution (Rigaud and Puppo, 1975).

Experimental Treatments and Harvest

Salt treatment of *V. faba* was started one week after inoculation with rhizobia. For each experimental group, 3 sets (4 pots per set) of plants were made: one set left without addition of NaCl in nutrient solution (control) and, in the second and third sets, NaCl was added to the nutrient solution reaching a final concentration of 60 and 120 mM, respectively. Plants were harvested at flowering stage (30 days after sowing) and washed out of the sand. Nodules were detached from their root and counted. After desiccation at 70°C during 72 hours, the dry matters of different parts of the plants (shoot, root and nodules) were quantified.

Mineral Nutrients Analysis

Total nitrogen content in plants was determined using 0.1 g of dry matter. The samples were analyzed by the Kjeldahl procedure as described by Lahrouni *et al.* (2013). Briefly, 0.5 g dry weight was mixed with 1 g of a catalyst mixture (K₂SO₄, CuSO₄.5H₂O and Se) and treated with 10 mL of sulfuric acid (98%). After mineralization, the volume was adjusted to 100 mL with distilled water; 40 mL of the

solution were transferred to Kjeldahl bottles containing few drops of NaOH (8N), and the resulting was distilled. The distillate was titrated with 0.05N sulfuric acid. The nitrogen content was expressed as mg of nitrogen per plant.

For determination of Na⁺, K⁺ and Ca²⁺, 0.5 g of plant shoot material (dry weight) was combusted at 550°C in a muffle furnace for 6 hours. Then the samples were soaked for 1 hour in 3 mL hydrochloric acid (5N), filtered and diluted with distilled water up to 25 mL. The cations were determined using a flame photometer (JeEN WOY PFP7). Concentrations were determined by comparison with calibration curves specific to each element.

Statistical Analysis

The experimental design was a randomized complete block. Growth attributes and nodulation parameters reported here are means of four replicates per treatment per strain. Data for nitrogen and micronutrients (Ca²⁺, Na⁺ and K⁺) content are the means of three replicates. All results were subjected to analysis of variance, with a Student-Newman-Keuls (SNK) method for the comparison of means using SPSS. Analysis Of Variance (ANOVA) was performed for comparison of means. ANOVA was used to test for differences in growth data, total nodule number per plant and mineral content ($P \leq 0.05$, SNK test). Standard Deviations (SD) were also calculated and are presented in the graphs.

RESULTS

Molecular Characterization of Rhizobial Strains

To analyze the taxonomic status of the strains RhOF15, RhOF34 and RhOF125, 16S rDNA was amplified with primers 16Sa and 16Sb. The primer parts were removed and the remaining sequence (1,398 bp) was used for phylogenetic analysis. For

comparison, corresponding sequences from different rhizobial strains were included. The phylogenetic tree (Figure 1) indicates that the strains are most similar to *Rhizobium leguminosarum*.

Effect of Salinity on Rhizobia-*V. faba* Symbiosis

Plant Growth Responses and Symbiotic Performance

Faba bean plants were grown at different salt concentrations and inoculated by tolerant or sensitive rhizobia strains in order to evaluate how these treatments affect their growth and symbiotic parameters. Growth parameters such as dry weight of the plant, number and dry weight of the nodules are given in Table 1, which clearly show the adverse effect of increased salinity on faba bean growth. After 30 days of salt treatment, the dry matter of shoots decreased significantly ($P < 0.05$) with increasing concentration of salinity. There was a significant difference in shoot biomass between plants exposed and not exposed to

salinity stress and between the tested rhizobia strains. At 60 mM of NaCl, the shoot biomasses were 0.53 and 0.51 g plant⁻¹ for the plants inoculated with salt tolerant strains RhOF34 and RhOF125, respectively, while they were significantly lower for the plants inoculated with the salt sensitive strain RhOF15 (0.44 g plant⁻¹) and for the non-inoculated plants (0.43 g plant⁻¹). At 120 mM NaCl treatment, the effect of salinity was more pronounced, except for the plants inoculated with the tolerant strain RhOF34 that kept shoot dry weight comparable to the 60 mM NaCl treatment (Table 1).

As for the root dry weights, there were generally no significant differences in the non-inoculated and the inoculated plants at 0 mM of NaCl. However, at 60 mM of NaCl, there was an increase of root dry weight in the inoculated plants as compared to the non-inoculated ones. The root dry weight was significantly higher in the presence of the tolerant rhizobial strains RhOF34 and RhOF125 under 60 mM salinity treatment. At 120 mM, the root dry weight was significantly higher in the plants inoculated with the salt tolerant strain (RhOF34) (Table 1).

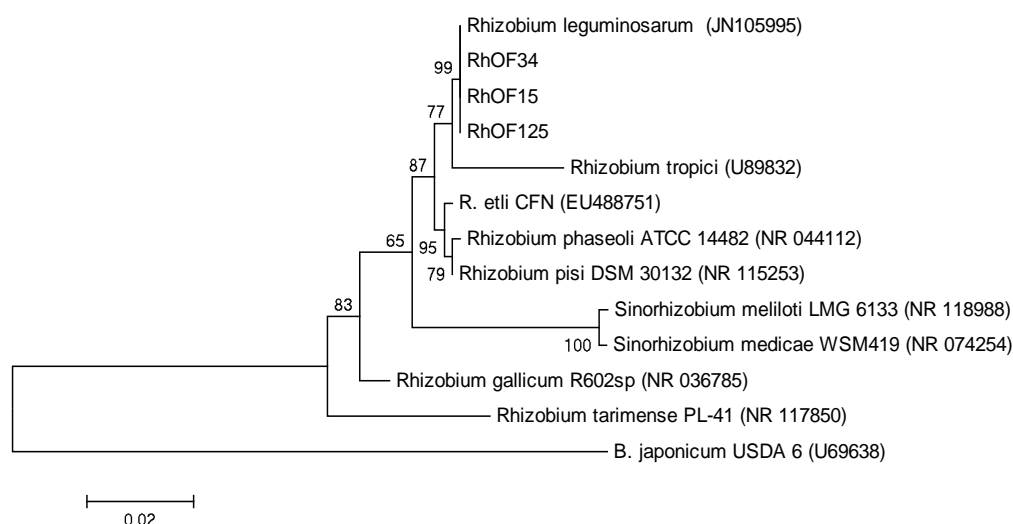


Figure 1. Maximum Likelihood phylogenetic tree based on *16S rDNA* gene sequences, showing the position of strains RhOF15, RhO34, and RhOF125 with regard to related genera of rhizobia. Bootstrap values based on 500 replications are given at branch points. Accession numbers are given in parenthesis. Scale bar, substitutions per nucleotide position.

**Table 1.** Effect of salt treatments on growth and nodulation parameters of *V. faba* inoculated with rhizobial strains. ^a

	Salt treatment	SDW ^b (g plant ⁻¹)	RDW ^c (g plant ⁻¹)	NN ^d (Number/Plant)	NDW ^e (g plant ⁻¹)
N ^a	0 mM	0.47 ± 0.02 e	0.26 ± 0.02 dc	-	-
	60 mM	0.43 ± 0.02 e	0.22 ± 0.02 f	-	-
	120 mM	0.34 ± 0.02 f	0.23 ± 0.03 f	-	-
RhOF34	0 mM	0.70 ± 0.03 a	0.23 ± 0.02 efd	63.50 ± 15.32 a	0.041 ± 0.006 a
	60 mM	0.53 ± 0.03 dc	0.34 ± 0.02 a	30.78 ± 17.20 a	0.029 ± 0.006 a
	120 mM	0.51 ± 0.04 d	0.33 ± 0.01 ab	01.33 ± 00.69 c	0.002 ± 0.003 c
RhOF125	0 mM	0.65 ± 0.03 b	0.25 ± 0.03 fedc	66.62 ± 15.67 a	0.038 ± 0.008 a
	60 mM	0.52 ± 0.03 d	0.31 ± 0.01 b	25.75 ± 12.90 ab	0.016 ± 0.003 b
	120 mM	0.36 ± 0.03 f	0.25 ± 0.01 efcd	01.33 ± 01.00 c	0.007 ± 0.002 bc
RhOF15	0 mM	0.59 ± 0.03 c	0.28 ± 0.01 c	43.28 ± 15.49 a	0.037 ± 0.004 a
	60 mM	0.44 ± 0.01 e	0.27 ± 0.01 efcd	11.11 ± 09.22 bc	0.009 ± 0.005 bc
	120 mM	0.35 ± 0.03 f	0.22 ± 0.02 ef	00.00 ± 00.00 d	0.000 ± 0.000 d

^a Non-inoculated plants; ^b Shoot Dry Weight; ^c Root Dry Weight; ^d Nodule Number, ^e Nodule Dry Weight. Values are means ± SD of four replicates. Mean followed with different letters in each column are significantly different at $P \leq 0.05$ according to the SNK test.

With increasing salinity, there was a sharp decrease in nodule number and nodule biomass. The plants exposed to different concentrations of salinity revealed a significant reduction ($P < 0.05$) in both nodule number and nodule dry weight. At 60 mM of NaCl, nodule dry weights were 29.87 and 15.45 mg for the plants inoculated with tolerant strains RhOF34 and RhOF125, respectively; and 8.95 for the plants inoculated with the sensitive strain RhOF15.

The number of nodules also decreased under 60 mM concentration of salt, it was reduced by 51.54, 61.35 and 74.32% for the plants inoculated with RhOF34, RhOF125 or RhOF15 strains, respectively. At the treatment of 120 mM NaCl, this reduction reached up to 97% for the faba bean plant inoculated with RhOF34 and RhOF125, whereas it was reduced by 100% for the plant inoculated with the sensitive strain RhOF15.

Nitrogen Content and Mineral Nutrients in Plant Tissues

Figure 2 illustrates the total nitrogen content in *V. faba* plants inoculated with

different rhizobia strains and treated with different concentrations of salt (0, 60 and 120 mM of NaCl). The total nitrogen content of the plants was affected by salinity and rhizobial inoculation. Indeed, at 60 mM, the nitrogen content was 20.23, 19.33 and 16.02 mg plant⁻¹ in the plants inoculated with RhOF34, RhOF125 or RhOF15 strains, respectively. At 120 mM NaCl treatment, the effect of salinity was more pronounced, except for the plants inoculated with the tolerant strain RhOF34 that kept the nitrogen content comparable to the 60 mM NaCl treatment (Figure 2).

In order to evaluate the effect of salinity on the variation of the nutritional balance in faba bean plants, the amounts of three micronutrients were evaluated (Na^+ , Ca^{2+} and K^+). The data in Figure 3-A reveal that Na^+ contents increased significantly ($P < 0.05$) in faba bean plants with increasing salt stress.

The Ca^{2+} concentration in the plants also increased under salt stress (Figure 3-B). At 120 mM NaCl, the plants inoculated with the sensitive strain (RhOF15) and the non-inoculated plants contained higher quantities

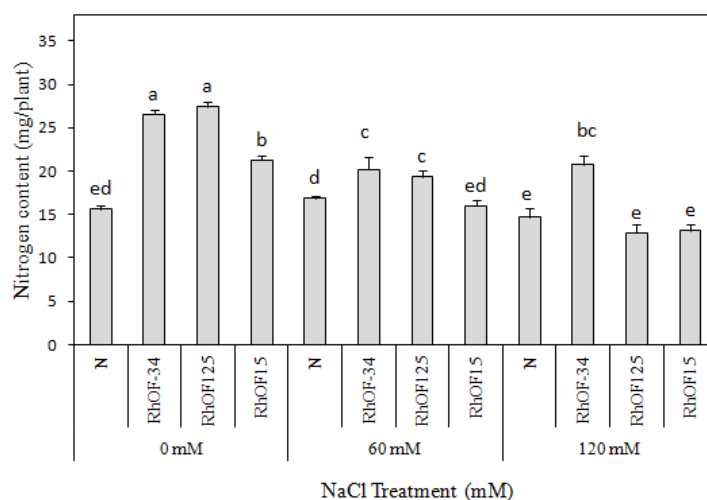


Figure 2. Effect of treatment with different concentrations of NaCl on nitrogen content in *V. faba* plants inoculated with rhizobial strains. N: Non-inoculated plants. Bars with different letters are significantly different at $P \leq 0.05$ according to the SNK test.

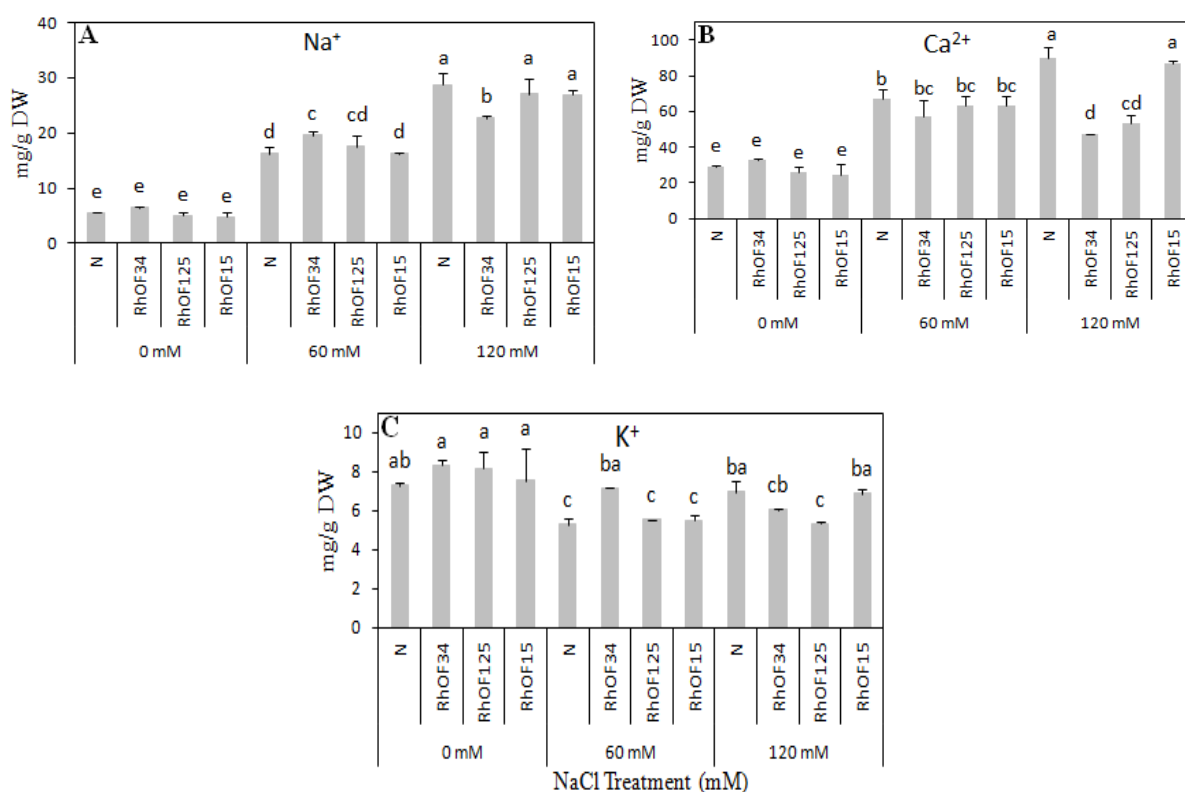


Figure 3. Mineral nutrient accumulation (Na^+ , Ca^{2+} and K^+) in shoots of *V. faba* inoculated with rhizobial strains. N: Non-inoculated plants. Bars with different letters are significantly different at $P \leq 0.05$ according to the SNK test.



of Ca^{2+} than the plants inoculated with the tolerant strains (RhOF34 and RhOF125).

As for K^+ , we noted a decrease in the plants exposed to 60 or 120 mM NaCl compared to the non-stressed plants. At 120 mM NaCl, the plants inoculated with the tolerant strains contained a lower concentration of K^+ than the plants inoculated with the sensitive strain or the non-inoculated plants (Figure 3-C).

DISCUSSION

Biological approaches using inoculation with osmotolerant and effective rhizobia can reduce the toxic effects of salinity stress against legume crops. Three *Rhizobium* strains identified as *Rhizobium leguminosarum* and with different tolerance against sodium chloride were used for *V. faba* inoculation. In general, our results indicate that salinity has a detrimental effect and leads to a significant drop in production of shoot dry matter compared to the control (0 mM). Comparable results were obtained for several legumes, such as *Vicia faba* (Cordovilla et al., 1999a), *Pisum sativum* (Cordovilla et al., 1999b), *Phaseolus vulgaris* (Faghire et al., 2011) and *Trifolium alexandrinum* (Ben Khaled et al., 2003). The reduction of shoot growth can be linked to disturbance of growth regulators (cytokinins and abscisic acid) induced by salinity (Kuiper et al., 1990; Sudhakar et al., 2001; Yurekli et al., 2004), or to a reduction of the photosynthetic capacity following a decrease in stomatal conductance of CO_2 under salt stress (Santiago et al., 2000). Otherwise, it should be noted that the effect of salinity on growth and productivity are not always negative. Low concentrations of NaCl (34 mM) in the medium led to a stimulation of shoot fresh weight and shoot dry weight with clover and alfalfa (Hussain et al., 1995; Ben Khaled et al., 2003). Abdul Qados (2011) demonstrated that shoot fresh weight of faba bean plants (Reina Mora variety) increased with 60 mM of sodium chloride.

In comparison to roots, the shoots were more affected by salinity. The development of the root system can be an adaptation of faba bean plants to salinity stress. Ben Khaled et al. (2003) noted that the resistance of the root system to salt stress might be due to a decrease of carbon allocation to leaves for the benefit of root growth.

It is known that nodule formation is highly sensitive to osmotic stress in rhizobia-legume symbiosis (Singleton and Bohlool, 1984; Velagaleti et al., 1990; Hassan and Eissain, 2013). Unsuccessful nitrogen fixation under salt stress may be partly due to a decrease in nitrogenase activity because saline conditions may reduce nodule respiration as a consequence of a limited O_2 conductance within the nodule (Serraj et al., 1995; Soussi et al., 2001). The rhizobial inoculation led to an increase of nodulation and nitrogen content of *V. faba* plants, especially those inoculated with the tolerant strain RhOF34 in salinity exposure. This result is in agreement with the finding of Hassan and Eissain (2013) showing that the inoculation with a salt tolerant mutant strain (not native) increased total nitrogen content in comparison with the inoculated plants by parental sensitive mutant.

Our results showed generally an increase of Na^+ and Ca^{2+} contents and a decrease of K^+ content with increasing NaCl concentration. This could be attributed to ionic imbalance, nutrient deficiency, and specific ion toxicity (Parida and Das, 2005). It is established that high levels of salt restrict plant production by nutrient imbalance due to the loss of nutrient uptake and/or transport to the shoot leading to ion deficiencies (Munns, 2002; Heidari and Jamshid, 2010). The Na^+ concentration in saline solution inhibits the uptake of K^+ , which might be explained by the competition between Na^+ and K^+ at the level of absorption sites (Cuin et al., 2009; Panda and Khan, 2009). Other experiments showed that an increase of Ca^{2+} uptake under salt stress was associated with the rise of Absciscic Acid (ABA) concentration (Chen et al., 2001). This might contribute to the

plant's capacity of regulating ion transport under high levels of external salinity (Chen *et al.*, 2001). In contrast, other authors reported that an increased Na^+ uptake is accompanied by a reduction in Ca^{2+} content and extra Ca^{2+} supply can inhibit the dominant transporters for Na^+ influx and improve membrane stability under salt stress (Patel *et al.*, 2011; Shores *et al.*, 2011; Semida *et al.*, 2014).

Interestingly, data in this article suggest that the inoculation with tolerant rhizobial strains reduced the contents of the analyzed ions in faba bean plants exposed to 120 mM NaCl. The application of salt tolerant rhizobia, especially RhOF34, reduces Na^+ , Ca^{2+} and K^+ absorption and protects plants against their toxic effects. In general, the obtained results are in agreement with the findings of Talaat *et al.* (2015) who noticed that the treatment with a mixture of beneficial microorganisms leads to a decrease of Na^+ and an increase of P, K^+ , Ca^{2+} and Mg^{2+} in *Phaseolus vulgaris* tissues, especially under saline conditions.

It was previously reported that trehalose production by rhizobia has a positive effect on plant growth and adaptation to abiotic stress of legumes (Suárez *et al.*, 2008). This solute is not common in vascular plants but it plays an important role as an osmoprotectant by stabilizing dehydrated membranes and enzymes and protecting biological structures under stress conditions (Dodd and Pérez-Alfocea, 2012). Inoculation of *Phaseolus vulgaris* L. subjected to 3 weeks of drought stress with *Rhizobium etli* overexpressing a trehalose-6-phosphate synthase gene (*otsA*) increased plant growth and grain yield in comparison with plants inoculated with *Rhizobium etli* wild-type strain (Suárez *et al.*, 2008). Also, microarray analysis of 7,200 expressed sequence tags from nodules of plants inoculated with the strain overexpressing the *otsA* gene revealed upregulation of genes involved in stress tolerance and carbon and nitrogen metabolism (Suárez *et al.*, 2008). Furthermore, Boscari *et al.* (2006) observed that inoculation of alfalfa with a

Sinorhizobium meliloti strain overexpressing the betaine transporter (*betS*), resulted in an increase of endogenous betaine in bacteroids and improved N_2 fixation of plants that were subjected to one week of salinization.

Saline conditions are among the major constraints for plant productivity. The isolation of effective and salt-tolerant rhizobial strains, to be used as inocula for the leguminous crop plants, could be an interesting strategy that may improve the yield of legumes under such stressful conditions. The obtained results showed that the native rhizobia strains isolated from Marrakech region soils are able to grow and effectively nodulate faba bean plants in salt stress conditions. Tolerant rhizobia including *R. leguminosarum* strain such as RhOF34, can increase the tolerance, the adaptation, and the protection of faba bean plant against salinity stress, and contribute to the enhancement in nutrient acquisition and improve the growth of inoculated *V. faba* plants. The role of selected rhizobia strains tolerant to salinity may be of great value and a friendly biotechnological pathway in order to improve faba bean productivity and tolerance towards salinity stress.

ACKNOWLEDGEMENTS

This work was financially supported by the project PMARS n°12/20 (Project BMBF n°01DH12051) and the Alexander von Humboldt foundation. The measurements of minerals were performed at the "Centre National d'Etudes et de Recherches sur l'Eau et l'Energie" (CNE-REE), Cadi Ayyad University, Marrakech, Morocco.

REFERENCES

1. Abdelmoumen, H., Filali-Maltouf, A., Neyra, M., Belabed, A. and El Idrissi, M. M. 1999. Effect of High Salts Concentrations on the Growth of Rhizobia and Responses to Added Osmotica. *J. Appl. Microbiol.*, **86**: 889-898.



2. Abdul Qados, A. 2011. Effect of Salt Stress on Plant Growth and Metabolism of Bean Plant (*Vicia faba*, L.). *J. Saud. Soc. For. Agric. Sci.*, **10**: 7-15.
3. Ahmed, S. 2009. Effect of Soil Salinity on the Yield and Yield Components of Mungbean. *Pak. J. Bot.*, **1**: 263-268.
4. Aragüés, R., Urdanoz, V., Çetin, M., Kirda, C., Daghari, H., Ltfi, W. and Douaik, A. 2011. Soil Salinity Related to Physical Soil Characteristics and Irrigation Management in Four Mediterranean Irrigation Districts. *Agr. Water Manage.*, **98**: 959-966.
5. Athar, H. R. and Ashraf, M. 2009. Strategies for Crop Improvement against Salinity and Drought Stress: An Overview. In: "Salinity and Water Stress: Improving Crop Efficiency", (Eds.): Ashraf, M., Ozturk, M. and Athar, H. R. Springer, Heidelberg, Germany, PP. 1-16.
6. Ben Khaled, L. B., Gómez, A., Honrubia, M. and Oihabi, A. 2003. Effet du Stress Salin en Milieu Hydroponique sur le Trèfle Inoculé par le *Rhizobium*. *Agronomie*, **23**: 553-560.
7. Borucki, W. and Sujkowska, M. 2008. The Effects of Sodium Chloride-Salinity upon Growth, Nodulation, Root Nodule Structure of Pea (*Pisum sativum* L.) Plants. *Acta Physiol. Plant*, **30**: 293-301.
8. Boscari, A., Van de Sype, G., Le Rudulier, D. and Mandon, K. 2006. Overexpression of *BetS*, a *Sinorhizobium meliloti* High-Affinity Betaine Transporter, in Bacteroids from *Medicago sativa* Nodules Sustains Nitrogen Fixation during Early Salt Stress Adaptation. *Mol. Plant. Microbe Interact.*, **19**: 896-903.
9. Brunel, B., Domergue, O., Maure, L., Brahic, P., Galiana, A., Josa, R. and El-Hajj, S. 2007. Potentialité des Associations Symbiotiques Plantes-Microorganismes pour Réhabiliter des Sites Fortement Dégradés en Milieu Méditerranéen. *Cah. Agric.*, **16**: 324-329.
10. Chen, S., Li, J., Wang, S., Hüttermann, A. and Altman, A. 2001. Salt, Nutrient Uptake and Transport, and ABA of *Populus euphratica*; a Hybrid in Response to Increasing Soil NaCl. *Tree*, **15**: 186-194.
11. Cordovilla, M. D. P., Ligerio, F., and Lluch, C. 1999a. Effect of Salinity on Growth, Nodulation and Nitrogen Assimilation in Nodules of Faba Bean (*Vicia faba* L.). *Appl. Soil Ecol.*, **11**: 1-7.
12. Cordovilla, M. D. P., Ligerio, F. and Lluch, C. 1999b. Effects of NaCl on Growth and Nitrogen Fixation and Assimilation of Inoculated and KNO₃ Fertilized *Vicia faba* L. and *Pisum sativum* L. Plants. *Plant Sci.*, **140**: 127-136.
13. Cuin, T. A., Tian, Y., Betts, S.A., Chalmandrier, R. and Shabala, S. 2009. Ionic Relations and Osmotic Adjustment in Durum and Bread Wheat under Saline Conditions. *Funct. Plant Biol.*, **36**: 1110-1119.
14. Dhaese, P., De Greve, H., Decraemer, H., Schell, J. and Van Mongatu, M. 1979. Rapid Mapping of Transposon Insertion and Deletion Mutations in the Large Ti-Plasmids of *Agrobacterium tumefaciens*. *Nucleic Acid. Res.*, **7**: 1837-1849.
15. Dodd, I. C. and Pérez-Alfocea, F. 2012. Microbial Amelioration of Crop Salinity Stress. *J. Exp. Bot.*, **63**: 3415-3428.
16. El-Hamdaoui, A., Redondo-Nieto, M., Torralba, B., Rivilla, R., Bonilla, I. and Bolaños, L. 2003. Influence of Boron and Calcium on the Tolerance to Salinity of Nitrogen-Fixing Pea Plants. *Plant soil*, **251**: 93-103.
17. Elsheikh, E. and Wood, M. 1990. Effect of Salinity on Growth, Nodulation and Nitrogen Yield of Chickpea (*Cicer arietinum* L.). *J. Exp. Bot.*, **41**: 1263-1269.
18. Faghire, M., Bargaz, A., Farissi, M., Palma, F., Mandri, B., Lluch, C., Tejera García, N. A., Herrera-Cervera, J. A., Oufdou, K. and Ghoulam C. 2011. Effect of Salinity on Nodulation, Nitrogen Fixation and Growth of Common Bean (*Phaseolus vulgaris*) Inoculated with Rhizobial Strains Isolated from the Haouz Region of Morocco. *Symbiosis*, **55**: 69-75.
19. Flowers, T. 2004. Improving Crop Salt Tolerance. *J. Exp. Bot.*, **55**: 307-319.
20. Hassan, M. M. and Eissain, R. A. 2013. Molecular Characterization of Salt Tolerant Rhizobial Strains Induced by Gamma Rays Using RAPD Markers. *New York Sci. J.*, **6**: 36-41.
21. Heidari, M. and Jamshid, P. 2010. Interaction between Salinity and Potassium on Grain Yield, Carbohydrate Content and Nutrient Uptake in Pearl Millet. *J. Agric. Biol. Sci.* **5**: 39-46.
22. Hussain, G., Al-Jaloud, A. A., Al-Shammary, S. F. and Karimulla, S. 1995. Effect of Saline Irrigation on the Biomass

- Yield, and the Protein, Nitrogen, Phosphorus, and Potassium Composition of Alfalfa in a Pot Experiment. *J. Plant Nutr.*, **18**: 2389-2408.
23. Johnson, M., Zaretskaya, I., Raytselis, Y., Merezuk, Y., McGinnis, S. and Madden, T.L. 2008. NCBI BLAST: A Better Web Interface. *Nucleic Acid. Res.*, **36**: 5-9.
 24. Jia, Y. and Gray, V. 2008. Growth Yield of *Vicia faba* L in Response to Microbial Symbiotic Associations. *S. Afr. J. Bot.*, **74**: 25-32.
 25. Kassem, M., Cappelano, A. and Gounot, A. M. 1985. Effet du Chlorure de Sodium sur la Croissance *In vitro*, l'Infectivité et l'Efficiencie de *Rhizobium meliloti*. *Mircen J. Appl. Microb.*, **1**: 63-75.
 26. Kumari, P. V., and Mesfin, Y. 2015. Testing Salt Tolerance to Boost on Chickpea (*Cicer arietinum* L. Mill Sp) Biomass/Cultivation. *Global J. Res. Med. Plant. Indigen. Med.*, **4**: 79-87.
 27. Kuiper, D., Schuit, J. and Kuiper, P. 1990. Actual Cytokinin Concentrations in Plant Tissue as an Indicator for Salt Resistance in Cereals. *Plant Soil*, **123**: 243-250.
 28. Lahrouni, M., Oufdou, K., El Khalloufi, F., Baz, M., Lafuente, A., Dary, M. and Oudra, B. 2013. Physiological and Biochemical Defense Reactions of *Vicia faba* L.-*Rhizobium* Symbiosis Face to Chronic Exposure to Cyanobacterial Bloom Extract Containing Microcystins. *Environ. Sci. Pollut. R.*, **20**: 5405-5415.
 29. MAPM, 2012. Situation de l'Agriculture Marocaine (Ministère de l'Agriculture et de la Pêche Maritime, Maroc). Available at: www.agriculture.gov.ma/pages/publications/situation-de-lagriculture-marocaine-n%C2%B010
 30. Munns, R. 2002. Comparative Physiology of Salt and Water Stress. *Plant Cell Environ.*, **25**: 239-250.
 31. Oufdou, K., Benidire, L., Lyubenova, L., Daoui, K., El Abidine Fatemi, Z. and Schröder, P. 2014. Enzymes of the Glutathione-Ascorbate Cycle in Leaves and Roots of Rhizobia-Inoculated Faba Bean Plants (*Vicia faba* L.) under Salinity Stress. *Eur. J. Soil Biol.*, **60**: 98-103.
 32. Panda, S. and Khan, M. 2009. Growth, Oxidative Damage and Antioxidant Responses in Greengram (*Vigna radiata* L.) under Short-Term Salinity Stress and its Recovery. *J. Agron. Crop Sci.*, **195**: 442-454.
 33. Paranychianakis, N. V. and Chartzoulakis, K. S. 2005. Irrigation of Mediterranean Crops with Saline Water: From Physiology to Management Practices. *Agric. Ecosyst. Environ.*, **106**: 171-187.
 34. Parida, A. K. and Das, A. B. 2005. Salt Tolerance and Salinity Effects on Plants: A Review. *Ecotoxicol. Environ. Saf.*, **60**: 324-349
 35. Patel, N. T., Vaghela, P. M., Patel, A. D. and Pandey, A. N. 2011. Implications of Calcium Nutrition on the Response of *Caesalpinia crista* (Fabaceae) to Soil Salinity. *Acta Ecol. Sinica*, **31**: 24-30.
 36. Ramoliya, P., Patel, H. and Pandey, A. 2004. Effect of Salinization of Soil on Growth and Macro-and Micro-Nutrient Accumulation in Seedlings of *Salvadora persica* (Salvadoraceae). *For. Ecol. Manage.*, **202**: 181-193.
 37. Rigaud, J. and Puppo, A. 1975. Indole-3-Acetic Acid Catabolism by Soybean Bacteroids. *J. Gen. Microb.*, **88**: 223-228.
 38. Santiago, L. S., Lau, T. S., Melcher, P. J., Steele, O. C. and Goldstein, G. 2000. Morphological and Physiological Responses of Hawaiian *Hibiscus tiliaceus* Populations to Light and Salinity. *Int. J. Plant Sci.*, **161**: 99-106.
 39. Semida, W., Taha, R., Abdelhamid, M. and Rady, M. 2014. Foliar-applied α -Tocopherol Enhances Salt-tolerance in *Vicia faba* L. Plants Grown under Saline Conditions. *S. Afr. J. Bot.*, **95**: 24-31.
 40. Serraj, R., Fleurat-Lessard, P., Jaillard, B. and Drevon, J. 1995. Structural Changes in the Innercortex Cells of Soybean Root Nodules Are Induced by Short-Term Exposure to High Salt or Oxygen Concentrations. *Plant Cell Environ.*, **18**: 455-462.
 41. Shrivastava P. and Kumar R. 2015. Soil Salinity: A Serious Environmental Issue and Plant Growth Promoting Bacteria as One of the Tools for Its Alleviation. *Saudi J. Biol. Sci.*, **22**: 123-131.
 42. Shores, M., Spivak, M. and Bernstein, N. 2011. Involvement of Calcium-Mediated Effects on ROS Metabolism in the Regulation of Growth Improvement under Salinity. *Free Radical Bio. Med.*, **51**: 1221-1234.



43. Singleton, P. W. and Bohlool, B. B. 1984. Effect of Salinity on Nodule Formation by Soybean. *Plant Physiol.*, **74**: 72-76.
44. Soussi, M., Khadri, M., Lluch, C. and Ocaña, A. 2001. Carbon Metabolism and Bacteroid Respiration in Nodules of Chick-Pea (*Cicer arietinum* L.) Plants Grown under Saline Conditions. *Plant Biosyst.*, **135**: 157-164.
45. Suárez R., Wong A., Ramírez M., Barraza A., Orozco M. C., Cevallos M. A., Lara M., Hernández G. and Iturriaga, G. 2008. Improvement of Drought Tolerance and Grain Yield in Common Bean by Overexpressing Trehalose-6-Phosphate Synthase in Rhizobia. *Mol. Plant. Microbe. Interact.*, **21**: 958-966
46. Sudhakar, C., Lakshmi, A. and Giridarakumar, S. 2001. Changes in the Antioxidant Enzyme Efficacy in Two High Yielding Genotypes of Mulberry (*Morus alba* L.) under NaCl Salinity. *Plant Sci.*, **161**: 613-619.
47. Sulieman, S. and Tran, L. S. P. 2015. Legume Nitrogen Fixation in a Changing Environment: Achievements and Challenges. Springer, **53**.
48. Talaat, N., Ghoniem, A., Abdelhamid, M. and Shawky, B. 2015. Effective Microorganisms Improve Growth Performance, Alter Nutrients Acquisition and Induce Compatible Solutes Accumulation in Common Bean (*Phaseolus vulgaris* L.) Plants Subjected to Salinity Stress. *Plant Growth Regul.*, **75**: 281-295.
49. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.*, **30**: 2725-2729.
50. Valizadeh, M., Moharamnejad, S., Ahmadi, M., and Mohammadzadeh Jalaly, H. 2013. Changes in Activity Profile of Some Antioxidant Enzymes in Alfalfa Half-Sib Families under Salt Stress. *J. Agr. Sci. Tech.*, **15**: 801-809.
51. Van Berkum, P. and Fuhrmann, J. J. 2000. Evolutionary Relationships among the Soybean Bradyrhizobia Reconstructed from 16S rRNA Gene and Internally Transcribed Spacer Region Sequence Divergence. *Int. J. Syst. Evol. Microbiol.*, **50**: 2165-2172.
52. Velagaleti, R., Marsh, S., Kramer, D., Fleischman, D. and Corbin, J. 1990. Genotypic Differences in Growth and Nitrogen Fixation among Soybean (*Glycine max*, L. Merr cultivars) Grown under Salt Stress. *Trop. Agr.*, **67**: 169-177.
53. Vincent, J. M. 1970. *A Manual for the Practical Study of Root Nodule Bacteria*. IBP Handbook, 15, Blackwell Scientific Publications, Ltd, Oxford, England.
54. Yurekli, F., Porgali, Z. B. and Turkan, I. 2004. Variations in Abscissic Acid, Indole-3-Acetic Acid, Gibberellic Acid and Zeatin Concentrations in Two Bean Species Subjected to Salt Stress. *Acta Biol. Cracov. Bot.*, **46**: 201-212.
55. Zahran, H. H. 1991. Conditions for Successful *Rhizobium*-Legume Symbiosis in Saline Environments. *Biol. Fert. Soil.*, **12**: 73-80.
56. Zahran, H. H. 1999 *Rhizobium*-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. *Microbiol. Mol. Biol. R.*, **63**: 968-989.
57. Zalidis, G., Stamatiadis, S., Takavakoglou, V., Eskridge, K. and Misopolinos, N. 2002. Impacts of Agricultural Practices on Soil and Water Quality in the Mediterranean Region and Proposed Assessment Methodology. *Agr. Ecosyst. Environ.*, **88**: 137-146.

اثر مایه کوبی *Rhizobium leguminosarum* روی رشد، جذب نیتروژن، و جذب و ادغام کانی ها در باقلا در تنش شوری

ل. بنیدیر، م. لحرونی، ف. الخلوفی، م. گوتفرد، و ک. آوفدو

چکیده

تنش شوری یکی از عمده ترین موانع محیطی محدود کننده تولید لگوم ها ست. هدف این پژوهش ارزیابی اثر تنش شوری (۰، ۶۰، و ۱۲۰ میلی مول NaCl) روی رشد، فرایند گره سازی، جذب نیتروژن و موجودی کانی های تغذیه در گیاه باقلا (*Vicia faba* L.) بود که با ریزوبیوم های محلی جدا سازی شده از ریشه گیاهان باقلا در مزارع منطقه Marrakech-Haouz مراکش مایه کوبی (تلقیح) شده بود. به این منظور، از سه ریشه *Rhizobium leguminosarum* شامل (RhOF34، RhOF125 و RhOF15) که در مقاومت به شوری تفاوت داشتند برای مایه کوبی باقلای فابا استفاده شد. نتایج نشان داد که اثر تنش شوری دراز مدت روی رشد و پارامترهای همزیستی *V. faba* متفاوت بود. در گیاهانی که باریسه حساس به شوری (RhOF15) مایه کوبی شده بودند، زیست توده (بیومس) شاخسار ها در تنش شوری کاهش یافت. در تیمار ۱۲۰ میلی مول نمک طعام، فرایند گره سازی روی ریشه باقلای فابا به شدت محدود شد، به ویژه در مورد ریشه حساس ریزوبیوم. با افزایش شوری، مقدار کل نیتروژن موجود در گیاهان آزمایشی کم شد، به استثنای گیاهانی که با ریشه مقاوم به شوری (RhOF34) مایه کوبی شده بودند و در آنها مقدار نیتروژن موجود در گیاه زیاد بود. با افزایش تنش شوری، غلظت سدیم و کلسیم در بافت های گیاهی افزایش شدیدی نشان داد در حالیکه غلظت پتاسیم کم شد. ریشه RhOF34 منجر به کاهش جذب Na^+ ، Ca^{2+} و K^+ در گیاه باقلا شد. نتیجه اینکه مایه کوبی با ریشه های RhOF125 و RhOF34 که به شوری مقاوم بودند منجر به افزایش زیست توده گیاهان، تعداد گره ها، و مقدار نیتروژن در گیاه شد و ظاهرا گیاه باقلای فابا را در برابر اثرات سمی شوری محافظت کرد.