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Phylogenetic and characterization of salt-tolerant rhizobial strain nodulating faba bean plants

Elsayed B. Belal[1]*, Hassan, M. M.[2,3] and El-Ramady, H. R.[4]

[1]Agricultural Botany Department (Agricultural Microbiology), Faculty of Agriculture, Kafrelsheikh University, 33516, Kafr El-Sheikh, Egypt.
[2]Genetics Department, Faculty of Agriculture, Minufiya University, Shebin El-Kom, Egypt.
[3]Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, KSA.
[4]Soil Sciences Department, Faculty of Agriculture, Kafrelsheikh University, 33516, Kafr El-Sheikh, Egypt.

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Improvement of faba bean production in the new reclamation land in Egypt requires isolation and selection of effective abiotic stress tolerant rhizobial strains. Three rhizobial strains were isolated from healthy faba bean plants growing in different geographic areas in Egypt. These isolates were adapted against different concentrations of NaCl (100, 150 and 200 mM) by using the enrichment method. They were evaluated by measuring the symbiotic N_2-fixation parameters under greenhouse and field conditions during two seasons (2010/2011 and 2011/2012). One rhizobial strain exhibited the highest values of symbiotic N_2-parameters, nitrogenase activity and proline content. Based on 16S rDNA and *nifH* gene sequence, this strain was shown to belong to the *Rhizobium leguminosarum* bv. *viciae*. A strong similarity was found between the 16S rDNA and *nifH* gene sequence of the strain E15 and *R. leguminosarum* bv. *viciae* 3841 (100% similarity for 16S rDNA and 95% similarity for *nifH* gene). The results show that the maximum growth of this strain was obtained at pH 7 and 30°C. This strain was tolerant to drought stress till 20% polyethylene glycol and it yielded the highest concentrations of indole-3-acetic acid (IAA) at the end of the logarithmic phase. This strain solubilized inorganic phosphorus. *R. leguminosarum* bv. *viciae* was able to survive, persist, grow and effectively nodulated faba bean plants at high salt concentrations under greenhouse and field conditions and it could be used for biofertilization to reduce the severe effects of salinity and drought stress in the new reclamation land in Egypt.

Key words: *Vicia faba*, *Rhizobium leguminosarum* bv. *viciae*, abiotic stress, *nifH* gene.

INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most important leguminous crops in Egypt. Its importance comes from the high value of seed protein content which is used for human and animal consumption. About 70% of human food comes mostly from cereals and legumes (FAO, 1999). In addition, it's a basic component of crop rotation in the Egyptian agriculture as it's one of excellent suppliers of soil nitrogen to the subsequent crops. The estimated average amount of N_2 - fixed by faba bean is 135 kg ha^{-1}, while it is 97 kg ha^{-1} for chickpea, 83 kg ha^{-1}

*Corresponding author. E-mail: elsayedb@yahoo.com. Tel: 0103169628.

Abbreviations: PCR, Polymerase chain reaction; **YEMA,** yeast extract mannitol agar; **IAA,** indole-3-acetic acid; **PEG,** polyethylene .glycol; **TCP,** tricalcium phosphate; **SI,** solubilization index; **NCBI,** National Center for Biotechnology Information

for lentil, 68 kg ha^{-1} for peanut and40 kg ha^{-1} for soybean for Egyptian conditions (Rizk, 1966). Elsheikh and Osman (2002) found that inoculation with rhizobial strains increased shoot dry weight after eight weeks by 21.4 and 29% compared to uninoculated plants under salt-stress. Rhizobial strains are very sensitive to soil environmental factors like high salt, drought, pH, and temperature stresses, which affect their dinitrogen fixation capacity and hence the productivity of legumes (Abdelmoumen et al., 1999).

Salinity stress is one of the most serious factors limiting the productivity of agriculture. The detrimental effects of salt on plants are a consequence of both a water deficit, resulting in osmotic stress, and effects of excess sodium ions on critical biochemical processes (Apse et al., 1999). Salt may affect symbiosis by its effects on the growth and survival of rhizobia in soil, restriction on root colonization, inhibition of processes of infection and nodule development, or impairment of active nodule functioning. The presence of high sodium chloride (NaCl) concentration has been reported to cause a reduction in the number of rhizobia in legume inoculants. Thus, tolerance to salt stress is an important part of saprophytic competence and competitiveness in rhizobia (Zahran, 1999).

In general, Egypt suffering from increasing population has both drought and arid climate, resulting in high soil salinity leading to tremendous losses in crop production. An essential aspect of the strategy to improve the yield of arid legumes in stressed environments must involve a combination of stress-tolerant cultivars and stress-tolerant rhizobia (Zahran, 1999; El-Nady and Belal 2005; Galvez, 2005; Essendoubi et al., 2007; Woldeyhannes et al., 2007; Sharma et al., 2013). Singleton et al. (1982) showed that rhizobium strains can grow and survive at salt concentrations which are inhibitory to most agricultural legumes.

Inoculation of such strains would enhance the nodulation and nitrogen fixing ability of the leguminous plants growing under saline conditions (Zahran, 1999; Ali et al., 2009). However, there are no previous reports on inoculation, characterization and surviving of free living soils rhizobia in the new reclamation land, Egypt. Therefore, the present investigation was designed to isolate, phylogenetic and characterize rhizobial strain nodulating faba bean plants against abiotic stresses including salinity and drought in the new reclamation land.

MATERIALS AND METHODS

Rhizobium isolation

Rhizobial isolates used through this present work were isolated from active nodules initiated on healthy faba bean plants collected from different regions (Kafr Elsheikh, Elbehira and Dakhlia Governorates) in Egypt according to Vincent (1970). *Rhizobium* like colonies were subjected to different cultural, biochemical, genetical and plant inoculation test for identification according to Somasegaran and Hoben (1985).

Enrichment culture and salinity tolerance test

Enrichment culture of *Rhizobium leguminosarum* bv. *viciae* capable of tolerating the different concentrations of sodium chloride (100, 150 and 200 mM) were established from the isolated rhizobial isolates according to the described method (El-Nady and Belal, 2005), then the tolerant single colony were picked up and maintained on the same medium to use in further studies.

Evaluation of the salinity tolerant isolates *in vivo*

In Leonard's Jars

Leonard's jars were used *in vivo* to evaluate the salinity tolerant isolates and determine the symbiotic N_2-fixation parameters (number of nodules/plant, fresh and dry weight of nodules / plant, N_2% and total N_2 in the shoot system) under sterilized conditions after 45 days from seed planting. Jars were filled with clean sand which had bean treated with 0.1 M HCl and washed several times with tap water and the Jars were autoclaved. Cultivar faba bean (Nobaria 1) was selected as recommended cultivar from National Program for leguminous crops - Agricultural Research Center, Ministry of Agriculture and Land Reclamation (Bulletin No. 747 of 2002) in Elbheira Governorate, Egypt. Seeds of cultivar faba bean (Nobaria 1) were surface-sterilized using standard methods (Somasegaran and Hoben, 1994). Seeds were sown (3 seeds / Jar) and inoculated by pippeting 5 ml of 10^8 cfu/ml rhizobial liquid culture around each seed. Jars were irrigated with sterilized water supplemented with 100, 150 and 200 mM NaCl and fertilized with nitrogen free nutrient solution prepared as described by Allen (1959). NaCl concentration was tested in the soil at two week's intervals in order to be adjusted. N_2% and total N_2 were assayed in the shoot by the Kjeldahl methods (AOAC 1990). Each treatment was represented by three replicates. Total nitrogen content = N_2% × dry weight of plants (El-Nady and Belal, 2005). *R. leguminosarum* biovar *viciae* strain was designated as E15 and was selected as efficient nitrogen fixer strain for further studies.

DNA extraction

Total DNA was extracted using the method of Sambrook et al. (1989). DNA (2 µl), approximately 50 ng, was used as template for the polymerase chain reaction assays. The amplifications were carried out in thermocycler as follows:

Preparation of polymerase chain reaction (PCR) reactions

2X PCR Master Mix from (Fermentas®, Lithuania) was purchased and used for PCR reaction. Each reaction contains all necessary reagents (dNTPs 200 nm of each and 0.6 unit of Taq DNA polymerase) except primers and DNA template for performing 25 µl reactions. 50 ng of genomic DNA and 100 p.mol of each primer were added and conditions used for 16S rDNA gene amplification were done according to the method of Shamseldin et al. (2009).

Analysis of amplified 16S rDNA genes

Primers described by Willems and Collins (1993), which correspond to *Escherichia coli* 16S rRNA gene positions 247 to 263 and 1291 to 1309, respectively, were used for PCR amplification of 16S rDNA genes. The presence of PCR products was ascertained by agarose (1% w/v) gel electrophoresis, at 100 V for 1 h.

16S rDNA sequencing

The 1512 bp 16S rDNA fragments were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and sequenced using primers 16S 1 and 16S 2 (Willems and Collins, 1993) on an Applied Biosystems model 373A DNA sequencer (Hambor City, Germany). The sequence reads were edited and assembled using the DNASTAR software (Lasergene, Madison, WI). BLAST searches were done using the NCBI server at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

Nif H gene

Plasmids extraction from bacterial strains was performed according to the manufacturers of Gene JET™ plasmid mini-prep kit (Fermentas). Then plasmid DNA was used for PCR reaction using Nif HF '5-TACGGCAACGGCGGCATCGGCAA-'3 as forward primer and Nif HR'5-AGCATGTCCTCGAGGTCCTCCA-'3 as reverse primer for Nif H gene according to Laguerre et al. (2001). The fragment obtained was sequenced as 16S gene.

Effect of pH and temperature on the growth of the tested strain

100 ml of YEM liquid medium was used to determine the effect of pH and temperature on growth of *R. leguminosarum* biovar *viciae* (E15). The medium was inoculated by 1 ml (10^8 cfu/ ml) of culture of *R. leguminosarum* biovar *viciae* (E15) strain. The experiments were carried out at pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5, and then the cultures were incubated on a rotary shaker at 30°C and 150 rpm/min for 5 days. To determine the optimum temperature, yeast extract mannitol liquid (YEM) medium at pH 7 was incubated at 20, 25, 30, 35 and 40°C and 150 rpm for 5 days. Cells number of the bacterial strain was determined by plating appropriate dilutions of liquid medium onto yeast extract mannitol agar (YEMA) medium.

Indole-3-acetic acid (IAA) production

IAA was obtained from 6 days at 30°C and 150 rpm cultures of *R. leguminosarum* biovar *viciae* (E15) in YEM liquid medium with 0.1 g/l tryptophan. After centrifugation (6000 rpm / 30 min), the liquid portion of an aliquot of liquid medium was used to determine the production of indole-3-acetic acid by the method described by Glickman and Dessaux (1995) and Elmahrouk and Belal (2007). The growth was determined as described above.

Effect of drought stress on *R. leguminosarum* bv. *viciae* (E15)

The effect of drought on growth of *R. leguminosarum* biovar *viciae* (E15) was investigated with polyethylene glycol (PEG) 6000. 50 ml YEM medium supplemented with 5, 10, 15 and 20% polyethylene glycol according to Abdel-Salam et al. (2010). YEM medium was inoculated by 1 ml from bacterial cell suspension at 10^8 cfu/ml. The cultures were incubated at 30°C and 150 rpm for 4 days. The growth was determined as described above.

Evaluation of the salinity tolerant isolates under field conditions

The experiment was carried out to evaluate faba bean cultivar response (Nobaria 1) for inoculation with *R. leguminosarum* biovar *viciae* (E15) in sandy soil under drip irrigation system. The experiment was carried out during winter season in Elbehira Governorate. This sandy soil (new reclamation land) was free from rhizobia. The treatments were divided into plots. Plots of 5 m long and 70 cm wide were sown with 150 faba bean seeds. It was applied at the time of planting as seed treatment. Peat-based rhizobia inoculum was prepared as described by Vincent (1970). The faba bean seeds of Nobaria 1 cultivar were inoculated according to El-Nady and Belal (2005) before sowing. Plots were sown with non-inoculated seeds and served as control treatments. Plots were irrigated with water supplemented with 200 mM NaCl; each treatment was represented by 4 plots as replicates. The inoculated plots with salt tolerant rhizobial strain were fertilized with mineral nitrogen fertilizer with rate of 25% N (31 kg/fed. of urea 46% N) as activation dose at the time of planting. The non-inoculated plots with salt tolerant rhizobial strain were fertilized with mineral nitrogen fertilizer at the rate of 100% N (125 kg/fed. of urea 46% N) at 0, 15, 30 and 45 days from sowing. The plots were fertilized with basal super phosphate (150 kg/fed.) before sowing. Each plot was fertilized with potassium sulphate (50 kg/fed.) at the time of flowering. NaCl concentration was tested in the soil two weeks intervals in order to be adjusted. The cultural practices, fertilization and pest control were carried out as commonly used. The symbiotic nitrogen fixation parameters were measured as described above.

Determination of nitrogenase activity

Nitrogenase activity was determined as C_2H_2 reduction (Serrano and Chamber, 1990). Nitrogenase activity was calculated from the integration of the chromatograph peaks with regard to a C_2H_4 standard curve.

Proline determination

Proline was determined in faba bean plants according to the method described by Bates (1973). Appropriate proline standards were included for calculation of proline in the sample. The proline content was detected also in *R. leguminosarum* bv. *viciae* (E15) cell. Cultures of strain *R. leguminosarum* bv. *viciae* (E15) were grown in YEM medium in the presence of 200 mM NaCl concentration to the late logarithmic phase. The cultures were harvested by centrifugation at 10 000 rpm for 15 min. The pellets were washed with 0.85% saline solution and re-centrifuged twice. The procedure of Prusiner et al. (1972) and Soussi et al. (2001) was followed to extract the hydrosoluble materials. The pellets were extracted in ethanol (70% v/v), centrifuged, and the supernatant fluid resuspended in water and stored at 20°C prior to analysis. The intracellular amino acid pools of cells were determined by the reaction with the ninhydrin reagent. Proline was determined as described above and a calibration curve was made using L-proline (Sigma). The supernatant was used for protein determination as described by Lowry et al. (1951) using bovine serum albumin as standard protein.

Solubilization of mineral phosphates

The phosphate solubilizing ability of *R. leguminosarum* biovar *viciae* (E15) was tested in Pikovskaya's medium (Pikovskaya, 1948) containing tricalcium phosphate (TCP) as insoluble phosphate source at 30°C and pH 7. Pikovskaya's medium was modified by adding to it Bromothymol blue. The formed halo zone surrounding the colony revealed phosphate solubilization and was expressed in solubilization index (SI) (Arun and Sridhar, 2005). Solubilization index (SI) = Diameter of the colony + Halo zone / Diameter of the colony (Sridevi et al., 2007). The strain was further tested in Pikovskaya's broth having initial pH 7. The cultures were incubated at 30°C and 150 rpm for seven days. After incubation period, the

final pH and phosphorus content of the medium were estimated in culture supernatant (Subba, 1993). From culture broth, insoluble materials were removed by filtering through whatman filter paper No.1 and the filtrate was centrifuged at 10000 rpm for 20 min. 10 ml of the filtrate was then mixed with 2.5 ml of Barton's reagent. The volume was adjusted to 50 ml with distilled water. After 10 min, the resultant color was read in a spectrophotometer at 430 nm.

Statistical analysis

The obtained data were subjected to the proper statistical procedures for analysis of variance according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Screening and isolation of the salinity tolerant rhizobial strains

The isolates were obtained from different geographical areas in Egypt. All the isolates including the tolerant salinity isolates were found to be motile, Gram negative, short rods. The colonies on YEMA were circular, non-spreading, non-chromogenic, easily emulsifiable in water and odourless. Streaking of the isolates on YEMA containing congo red or bromothymol blue (BTB) gave normal reactions. Fast growing rhizobia such as *R. leguminosarum* bv. *viciae* have an acid reaction, turning the medium yellow and also it does not absorb Congo red when plates are incubated in the dark. The formed zone in litmus milk gave alkaline reaction. The growths of these isolates on yeast extract mannitol broth medium showed uniform turbidity.

Symbiotic performance of the rhizobial isolates in Leonard Jars

Data presented in Table 1 shows the symbiotic performance of the isolated rhizobial isolates under non or saline stress with adapted or non adapted strains. A significant positive correlation was found between the salt tolerance and the adaptation of rhizobial strains in salinity soil. Under salinity stress, in general, nodulation and the other N_2-Fixiation parameters were highly affected by different concentrations of sodium chloride with intolerant (original) rhizobial isolates (without enrichment) and were retarded with increasing salt concentration compared with non-salinity stress. On the other hand, the N_2-Fixiation parameters were higher with the tolerant rhizobial isolates than with intolerant isolate. The results showed no nodules were formed on non-inoculated faba bean plants. However, inoculation with different isolates of *R. leguminosarum* bv. *viciae* showed variation in nodulation and this reflect their ability to nitrogen fixation. All rhizobial isolates showed significant increase in nodulation parameters compared with uninoculated plants. All rhizobial isolates induced also significant increase in number and dry weight of nodules per plant compared with uninoculated plants grown in normal and saline soil. All biofertilizer inoculants induced significant increase in dry weight of shoot per plant at 45 days from plant sowing compared with uninoculated plants. Data presented in Table 1 shows significant differences in total N_2 mg / plant at 45 days plant-old. The results revealed inoculation with salinity tolerant rhizobial isolates increased significantly the total N_2 mg / plant in saline sandy soil compared with the inoculation with salinity un-tolerant rhizobial isolates.

The results shown in Table 2 demonstrate the efficiency of these salt tolerant isolates (E15, M13 and M14) in Leonard jars. Both nitrogenase activity and proline content were increased in faba bean plants which were inoculated with adapted rhizobial isolates in comparison with the non-inoculated plants. Therefore, inoculation in the presence of salt stress with the salinity tolerant rhizobium strains did help the faba bean plants to increase proline content and nitrogenase activity under saline conditions. The results shown in Table 2 demonstrate that among three strains, we found a high degree of diversity. One strain was highly tolerant to a salt concentration 200 mM NaCl (E15). In the present study, the salt induced a marked accumulation of proline, and the content of this solute was highest at 200 mM. The highest dosages of salt diminished proline levels, indicating that these compounds may contribute to osmoregulation within salinity ranges tolerated by *R. leguminosarum* bv. *viciae* (E15, M13 and M14). The *R. leguminosarum* bv. *viciae* strain E15 was efficient according to its N_2-fixiation parameters and was selected for further experiment.

These morphological and colony characters were confirmed to be *Rhizobium* type as defined by Somasegaran and Hoben (1985). To select the efficient N_2-fixing salinity tolerant faba bean rhizobial isolates, screening experiment was carried out in Leonard Jars system (Vincent, 1970; Somasegaran and Hoben, 1985). It is well known that the host plant inoculation by native strains with high efficiency has a positive effect on plant yield and biological nitrogen fixation process (Jebera et al., 2000; Rehman et al., 2002; Shamseldin et al., 2009; Fahmi, et al., 2011).

This indicate that salinity tolerant rhizobial isolates could tolerate soil salinity and increased nodulation parameters. This results agree with Miller and Wood (1996), El-Nady and Belal (2005) and Fahmi et al. (2011), who reported that soil salinity adversely affects nodulation and nitrogen fixation capacities of rhizobia, resulting in lower productivity of legumes. In addition, they reported that no nodulation was observed in saline soil. Growth of most rhizobial strains was inhibited by 100 mM NaCl, while some strains could tolerate more than 300 mM concentrations of NaCl. The obtained results illustrated also that all biofertilizer inoculants showed significant increase in nodulation parameters compared with uninoculated plants. This agreed with Dadarwal and Sen (1974), who found that both nodulation and yield increased significantly

Table 1. Effect of different concentrations of NaCl on symbiotic N_2-fixing parameters of faba bean plants inoculated with two isolates of *Rhizobium leguminosarum* bv. *viciae* in Leonared Jars at 45 days from planting*.

Treatment	Concentration	Number of Nodules/plant	D.W. of nodules/plant (g)	D. W. of shoots/plant (g)	% N_2	Total N_2 mg/plant
Isolate E15 (enirched)	0 NaCl	115^{a}	0.4^{a}	2.4^{a}	1.7^{a}	40.8^{a}
	100mM	92b^{c}	0.28^{b}	2.2^{bc}	1.3^{b}	28.6^{b}
	150mM	79^{c}	0.23^{c}	1.9^{bcd}	1.1^{d}	20.9^{c}
	200mM	61^{d}	0.16^{d}	1.6^{de}	1^{e}	16^{d}
Isolate E15 (Non-enirched)	100mM	29^{e}	0.030^{e}	1.0f^{g}	0.3^{g}	3.00^{f}
	150mM	9f^{g}	0.002^{e}	0.7^{gh}	0.1^{i}	0.70^{f}
	200mM	4^{g}	0.001^{e}	0.4^{hi}	0.01^{i}	0.04^{f}
Isolate M13 (enirched)	0 NaCl	98^{ab}	0.3^{a}	2^{ab}	1.5^{b}	30^{b}
	100 mM	88^{bc}	0.21^{b}	1.5^{cde}	1.20^{c}	18^{c}
	150 mM	76^{c}	0.17^{c}	1.30^{e}	1.0^{e}	13.0^{d}
	200 mM	56^{d}	0.11^{d}	1.10^{f}	0.62^{f}	7.2^{e}
Isolate M14 (Non- enirched)	100 mM	21^{ef}	0.022^{e}	0.80^{fg}	0.20^{h}	1.60^{f}
	150 mM	9^{fg}	0.002^{e}	0.46^{h}	0.10^{i}	0.50^{f}
	200 mM	4^{g}	0.001^{e}	0.29^{i}	0.01^{i}	0.03^{f}
Isolate M14 (enirched)	0 NaCl	98^{ab}	0.3^{a}	1.9^{ab}	1.51^{b}	28.7^{b}
	100 mM	88^{bc}	0.21^{b}	1.5^{cde}	1.20^{c}	18^{c}
	150 mM	76^{c}	0.17^{c}	1.30^{e}	1.0^{e}	13^{d}
	200 mM	56^{d}	0.11^{d}	1.10^{f}	0.62^{f}	6.82^{e}
Isolate M14 (Non-enirched)	100 mM	21^{ef}	0.022^{e}	0.78^{fg}	0.19^{h}	1.50^{f}
	150 mM	9f^{g}	0.002^{e}	0.43^{h}	0.10^{i}	0.43^{f}
	200 mM	4^{g}	0.001^{e}	0.28^{i}	0.01^{i}	0.03^{f}
Non Inoculated	No NaCl	0	0	0.3^{i}	0.1^{i}	1.2f^{g}

ans are value of three replications; Means followed by the same letter in a column are not significantly different but by different letters are significantly different (P= 0.05) using the Duncan multiple range test.

Table 2. Effect of salt stress on nitrogenase activity of rhizobial isolates and proline accumulation in faba bean plants*.

Isolate	Nitrogenase activity (µM C_2H_4/dry weight nodules)					Proline accumulation (µM/ g plant)				
	0 mM	50 mM	100 mM	150 mM	200 mM	0 mM	50 mM	100 mM	150 mM	200 mM
M13	6.2^{c}	4.8^{c}	2.4^{c}	1.7^{c}	1.01^{c}	92^{b}	114^{bc}	295^{c}	545^{b}	^{b}622
M14	12.1^{b}	10.3^{b}	9.3^{b}	8.1^{b}	7.9^{b}	89^{c}	119^{b}	296^{c}	573^{c}	^{c}660
E15	18.4^{a}	16.2^{a}	13.1^{a}	11.1^{a}	10.1^{a}	106^{d}	198^{d}	273^{b}	591^{d}	^{d}708
Control	**ND	**ND	**ND	**ND	ND**	30^{a}	45^{a}	60a	75^{a}	150^{a}

Means are value of three replications; Means followed by the same letter in a column are not significantly different but by different letters are significantly different (P= 0.05) using the Duncan multiple range test. ND** means not detected.

due to inoculation. Inoculation of stress tolerant rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress conditions. The ability of legume hosts to grow and survive in saline condition is improved when they are inoculated with salt tolerant strains of rhizobia. These results agreed with EL-Nady and Belal (2005), Ali et al. (2008) and Fahmi et al. (2011) who found that inoculation with stress tolerant rhizobia may enhance nodulation and nitrogen fixation ability of plants under stress condition. *Rhizobium*- legume symbioses

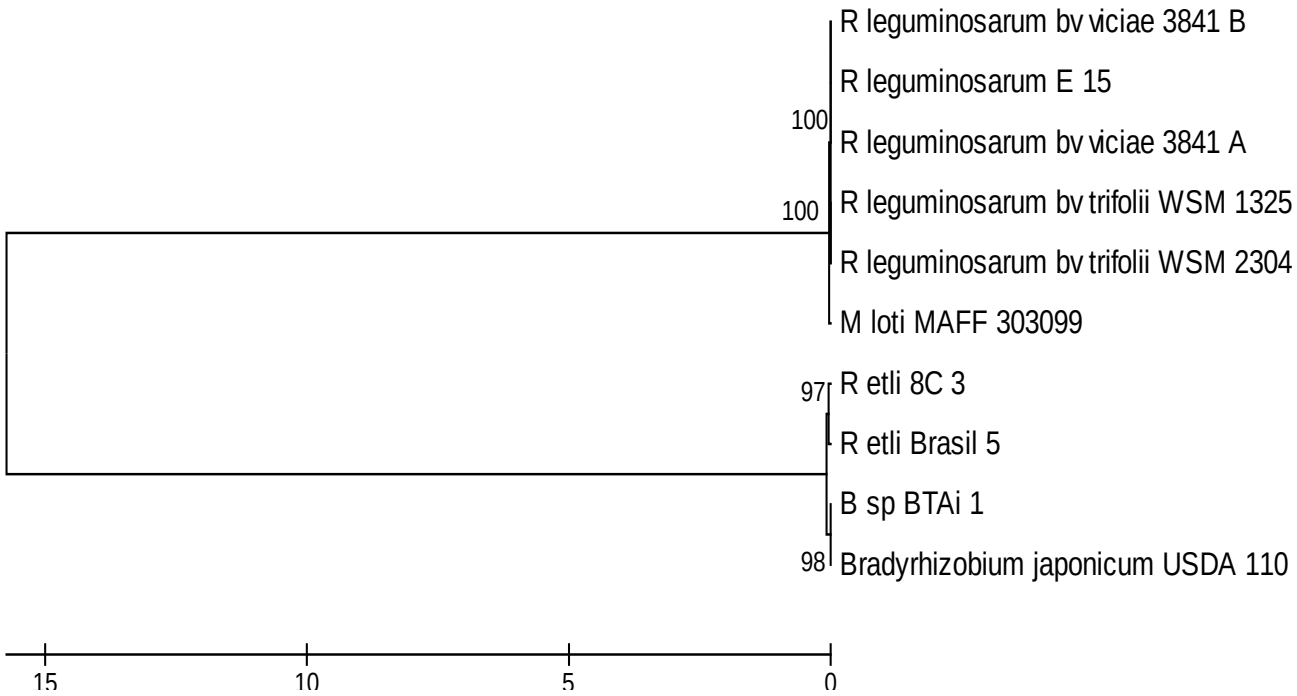

Figure 1. Dendogram illustrating the genomic relationship among ten isolates belonging to genus *Rhizobium* revealed by UPGMA cluster analysis.

are important for their nitrogen input, but salinity and elevated temperature in arid and semi-arid areas limit their effectiveness, and therefore plant growth and productivity.

It is suggested that the rhizobia sensitivity to salinity may be partly responsible for the inhibition of nitrogen fixation by faba bean under salinity stress. Also, this could be mostly due to the high osmotic pressure of the nutrient solution and lack of aeration resulted by mal formation of the soil structure, with regarding to sodium cations in the rhizosphere region. These results find confirmation with those observed by Sirry et al., (1980). The behavior of nitrogen-fixing bacteria under severe environmental conditions such as salt stress, drought was reviewed by Zahran (1999). This also agreed with Bolaños et al. (2003), who found that nodules developed in *Pisum sativum* inoculated with *R. leguminosarum* bv. *vicae* 3841 and growing under saline conditions (75 mmol/l. NaCl) were non functional and had abnormal structure .Isolate E15 had highest value in N_2-Fixiation parameters than the isolates M13 and M14.

A number of nitrogen-containing compounds accumulate in plants exposed to saline stress. The most frequently accumulating nitrogen-containing compounds include amino acids, amide, proteins, quaternary ammonium compounds, and polyamines. The specific nitrogen containing compounds that accumulate in saline environments vary with plant species. Many plants accumulate proline as a nontoxic and protective osmolyte under saline conditions (Jain et al., 2001).

Identification of *Rhizobium leguminosarum* bv. *viciae* (E15) using 16S rDNA

This bacterial strain (E15) was identified according to previous described morphological, physiological, plant test in Leonard jars as well as using analysis of 16S rDNA (Figure 1). According to the 16S rDNA analysis, the phylogenetic tree of the isolated bacteria (E15) and related bacterial species based on the 16S rDNA sequence is shown in Figure 1. It can be clearly seen that the isolated bacteria was included in the genus *Rhizobium* and closely related to the species *R. leguminosarum*. It showed the highest sequence similarities with *R. leguminosarum* bv. *viciae* 3841 A (100%), and *R. leguminosarum* bv. *viciae* 3841 B (100%).

Analysis of the *nif H* DNA region

Data presented in Figures 2 and 3 show that the *nifH* DNA region of *R. leguminosarum* bv. *viciae* strain E15 was located and sequenced as a first step towards identifying the regulatory DNA elements involved in Nif H expression in this symbiotic bacterium. A gene aliment was screened with *orf71* that was generated from *nif H* available at National Center for Biotechnology Information (NCBI) data base from strains *R. leguminosarum* bv. *viciae* 3841 with accession number NC_008381.1. Identities were 743 of 782 nucleotides about 95% max identification, query coverage 99% and

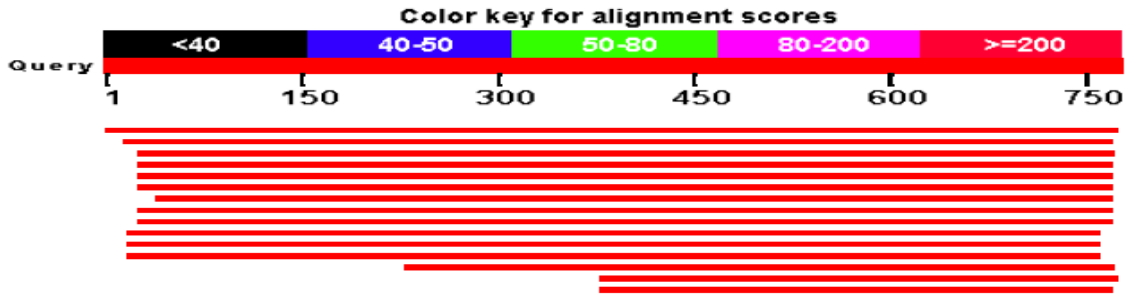

Figure 2. NCBI database aliment of nucleotide sequences of the region of *nif H* gene in *Rhizobium leguminosarum bv. viciae* E15 with different rhizobial isolates.

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Sbjct  161749  CACTGGGCAGCTTTCAACTTGGCATCCCTGGCTTGAAGT-TCGGCAAGCATCTGCTCGTC  161807

Query  62      GGTCTTCATGATCCCG-AATACGAGAATTAATGTCCTCAAGCTCTTCCATGGTGCCTGAA  120
Sbjct  161808  GGTCTTCATGATCCCGAAAT-CGAGAA-GCATGTCCTCAAGCTCTTCCATGGTG-ATG-G  161863

Query  121     GGGTCGGGATGGTGCCTTTGCCAGAATTGTCATGTATTCTCTGGGCAAGTG-TCGGGTAC  179
Sbjct  161864  GGGTCGGGATGGTGCCTTTGCCAGAATTGTCATGTATTCTCTGGGCAAGTGTTC-GGTAC  161922

Query  180     TCGGCCGCTTGCTGAGAGTCCGGCGCATATTGGATCACCGTCATCTTTCTAAGCTCGGCG  239
Sbjct  161923  TCGGCCGCTTGCTGAGAGTCCGGCGCATATTGGATCACCGTCATCTTTCTAAGCTCGGCG  161982

Query  240     TGTTGGACAATG--CCCGGGTGGCACGAAATGGTTGAGCTTAGAATTGAGTTAGGCAGCC  297
Sbjct  161983  TGTTGGACAATGTTGTCGCGTGGCACGAAATGGATGAGCTTAGAATTGAGTTTGGCAGCC  162042

Query  298     AGCGCTTCGGCGAGGTCGAGTTTCCGATC-ATCGGTTTGACGCAACGTTACAAATGA--C  354
Sbjct  162043  AGCGCTTCGGCGAGGTCGAG-TT-CG--CGATCGGTTTGACGC-TCGTTACAAATGAGGC  162097

Query  355     CGCCCAGGCGTACGCTGCCG-C--CAGCATATTTCAGGATGCCCCTGGCGTTATTGTTG  411
Sbjct  162098  CGCCCAGGCGTACGCTGCCGCCTGCAGCATATTTCAGGATGCCCCTGGCG-ATATTGTTG  162156

Query  412     GCGGCATAGAGCGCCATCATCTCGCCGGACATGACGATATAAATTTCCTGAGCTTTGTTT  471
Sbjct  162157  GCGGCATAGAGCGCCATCATCTCGCCGGACATGACGATATAAATTTCCTGAGCTTTGTTT  162216

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Sbjct  162217  TCACGGATCGGCATCGCGAAGCCGCCGCACACAACGTCACCGAGCACGTCATAAGACACG  162276

Query  532     TAGTCGACATCGTTGTAGGCGCCGTTCTCCTCGAGAAAGTTGATCGACGTGATAACGCCG  591
Sbjct  162277  TAGTCGACATCGTTGTAGGCGCCGTTCTCCTCGAGAAAGTTGATCGACGTGATAACGCCG  162336

Query  592     CGTCCCGCGCAGCCGACGCCTCCCTCCGGGCCGCCAGACTCCACGCATTTGATACCTTTA  651
Sbjct  162337  CGTCCCGCGCAGCCGACGCCTGGCTCCGGGCCGCCAGACTCCACGCATTTGATACCTTTA  162396

Query  652     TAGCCTACCTTGAGC-CTATCTTCGAGTTCAAGATCTTCAACCGAACCTCTCGTTGCAGC  710
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Sbjct  162456  GAGATCAAGAACCGTATCCTGGGCCTTCGAGTTCAAGATCAGGCGCGTAGAATCGGCCTT  162515
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Figure 3. Comparison of nucleotide sequences of the region of *nif H* gene in *Rhizobium leguminosarum* bv. *viciae* E15 (top line) with or f5-nif H *Rhizobium leguminosarum* bv. *viciae* 3841 (bottom line). The numbers on the left indicate the positions of the first nucleotide according to the NCBI accession no. NC_008381.1.

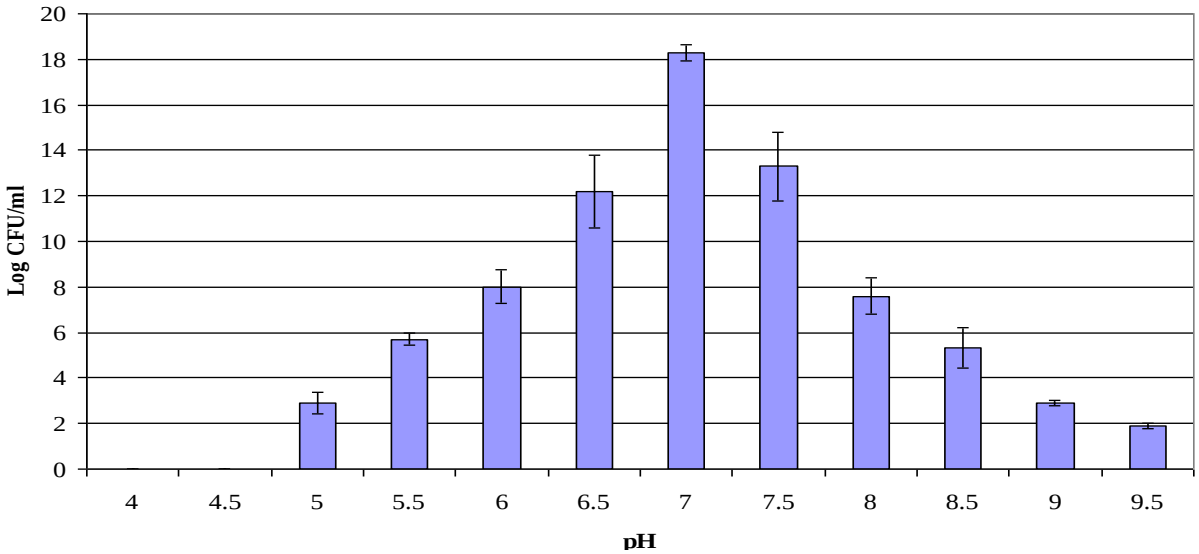

Figure 4. Effect of pH on growth of *Rhizobium leguminosarum* bv. *viciae* E15.

gap 3% .

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 31.62125433 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 920 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). These results agree with those of Groenger et al. (1987).

Effect of pH and temperature on growth of *R. leguminosarum* bv. *viciae* E15

Data presented in Figures 4 and 5 show the optimum pH and temperature for growth of *R. leguminosarum* bv. *viciae* E15 in YEM liquid medium. The optimum pH and temperature were 7 and 30°C, respectively. *R. leguminosarum* bv. *viciae* E15 exhibited a wide diversity in their different pH tolerance, where it grew from acidic pH 5 to alkalinity pH 9.5 (Figure 4) whereas, this strain did not exhibit any growth at pH 4 and 4.5. Our results were in agreement with Sadowesky et al. (1983). Harun et al. (2009) found also that lentil nodulating rhizobia can grow at acidic and alkaline pH. There might be a relation between pH of origin of isolated and their acid and alkaline pH tolerance. Temperature conditions have a great effect on rhizobial growth and symbiotic performance (Zahran, 1999). As shown in Figure 5, maximum growth of *R. leguminosarum* bv. *viciae* E15 was obtained at 30°C whereas, this strain did not grow at 40°C. Harun et al. (2009) observed that all lentil nodulating rhizobial strains grew well at 33°C and one strain could grow at 38°C.

IAA production

Faba bean nodulating rhizobia showed very interesting characteristics such as auxin (IAA) production (Figure 6). Data presented in Figure 6 illustrates that IAA formation started when the strain grew on the medium supplemented with tryptophan. The maximum accumulation of IAA occurred at the end of logarithmic phase, and after that, the accumulation of IAA decreased at the beginning of the stationary growth phase. IAA accumulation coincided with increase in the specific growth rates of the cultures. IAA accumulation coincided with increase in the specific growth rates of the cultures. Several studies reported that different strains of *R. leguminosarum* bv. *viciae* are endowed with these characteristics (Etesami et al. 2009; Jida and Assefa, 2011).

Solubilization of mineral phosphates

The isolated *R. leguminosarum* bv. *viciae* from faba bean plants was fast growing as it produced acid in YEM broth and colony diameter on YEMA was 3 mm. Fast growing rhizobia such as *R. leguminosarum* bv. *viciae* (E15) have an acid reaction, turning the medium yellow (YEMA supplemented with bromothymol blue). Faba bean nodulating rhizobia showed very interesting characteris-

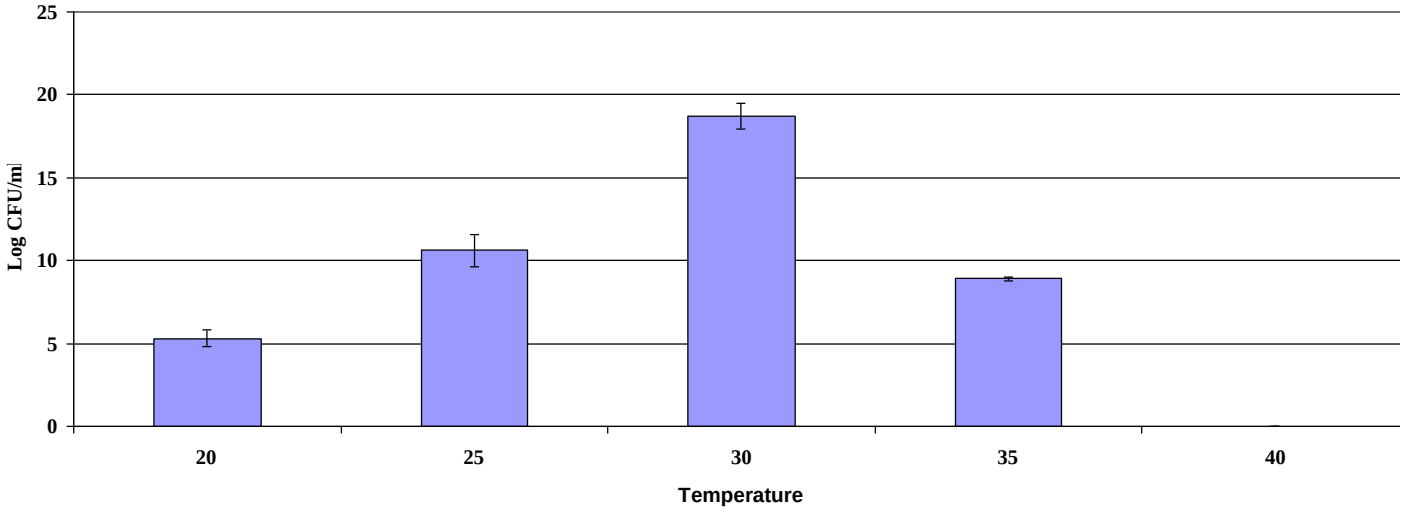

Figure 5. Effect of temperature on growth of *Rhizobium leguminosarum* bv. *viciae* E15.

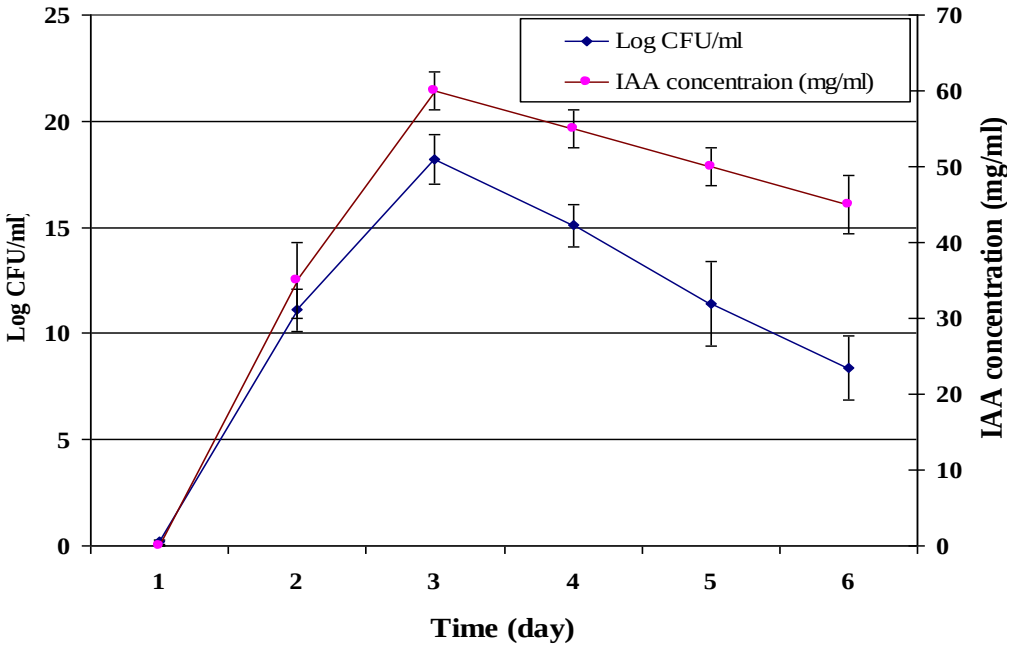

Figure 6. IAA production by *Rhizobium leguminosarum* bv. *viciae*. (E15) starin in submerged culture with tryptophan.

tics such as inorganic phosphate solubilization (Table 3). This strain formed clear zone around the colony on Pikovskaya's s agar medium after 3 days of inoculation and it gradually increased up to 7 days. The colony diameter is almost similar throughout the incubation period. Solubilization index (SI) of this strain ranged between 2.67 and 3.33 (Table 3). In liquid medium, maximum solubilization was also recorded (640 μgml^{-1}). A fall in pH accompanied phosphate solubilization, due to production of organic acids observed up to seven days.

It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings. The production of organic acids by phosphate solubilizing bacteria has been well documented. Among organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid which is present in *R. leguminosarum* and *Rhizobium meliloti* (Duff and Webley, 1959). Halder et al. (1990) showed

Table 3. Solubilization of tricalcium phosphate by *Rhizobium leguminosarum* bv. *viciae* (E15).

Strain	Incubation time (day)	Colony diameter (mm)	Zone of solubilization (mm)	Solubilization index (SI)	Final pH of medium	P-liberated (μgml^{-1})
Rhizobium leguminosarum bv. *viciae*	3	3	5	2.67	6.2	622
	5	3	7	3.33	5.8	630
	7	3	7	3.33	5.6	640

*SI, Solubilization index; pH, final pH of medium.

Table 4. Growth of *Rhizobium leguminosarum* bv. *viciae* E15 strain in YEM medium under different drought stress.

Growth	YEM liquid medium supplemented with PEG 6000				
	0	5%	10%	15%	20%
Log CFU/ml	18.4	12.1	8.2	4.2	2.5

that the organic acids isolated from a culture of *R. leguminosarum* solubilized an amount of P nearly equivalent to the amount that was solubilized by the whole culture. Goldstein and Liu (1987) have proposed that the direct periplasmic oxidation of glucose to gluconic acid, and often 2-ketogluconic acid, forms the metabolic basis of the mineral phosphate solubilization phenotype in some Gram negative bacteria.

Alternative possibilities other than organic acids for mineral phosphate solubilization have been proposed based on the lack of a linear correlation between pH and the amount of solubilized P. Other mechanisms have been considered, such as the production of chelating substances by microorganisms as well as the production of inorganic acids, such as sulphidric, nitric, and carbonic acid. However, the effectiveness of these processes has been questioned and their contribution to P release in soil appears to be negligible (Rodríguez, 1999).

Proline determination in *R. leguminosarum* bv. *viciae* (E15) cell

The total amino acid content increased significantly with salinity (data not shown) and the highest concentration was reached in cells grown with 200 mM NaCl. High proline content was found in this rhizobial strain, representing up to 25% of total protein content. High proline content was found in this rhizobial strain, representing up to 25% of total protein content. It is noteworthy that salt at 200 mM boosted proline accumulation up to twofold compared with control cells. Our results are in agreement with those of Soussi et al. (2001). A significant positive correlation was found between the salt tolerance and the adaptation of rhizobial strains in drought conditions. Strain tested in this study also seemed to be well adapted to high or low pH. These findings confirm previous reports with rhizobial strains from other areas (Kulkarni et al., 2000; Abdel-Salam et al., 2010), who found that all strains tolerating salt concentrations were highly resistant to water potential conditions.

Determination of drought-tolerant efficiency

In the present study, rhizobial strain growth were measured after exposure to 5 or 20% PEG 6000, for four days (Table 4). The growth decreased in retard with increasing PEG 6000 concentration. Strain tested in this study also seemed to be well adapted to high or low water potential (Table 4). A significant positive correlation was found between the salt tolerance and the adaptation of rhizobial strains in drought conditions. Strain tested in this study also seemed to be well adapted to high or low pH.

Evaluation of the salinity tolerant isolates under field conditions

Based on the result of our studies, the following strain *R. leguminosarum* bv. *viciae* E15 is highly recommended for field trail and ecological competitiveness studies under Egyptian soils especially in new reclamation land in Elbehira Governorate, Egypt. This strain have exhibited interesting features such as wide range of tolerance of salinity, drought, pH and temperature, auxin (IAA) production, proline accumulation, inorganic phosphate solubilization and highly effective nitrogen fixation. Results in Table 5 and Figure 7 confirm the efficiency of this salt resistances strain (E15) under aseptic conditions. Seed treatment with peat-based preparation of strain (E15) at the time of planting in sandy soil under field con-

Table 5. Effect of inoculation with salt-tolerant *Rhizobium leguminosarum* bv. *viciae* on symbiotic N_2-fixing parameters and praline accumulation in faba bean plants under field conditions.

Treatment	Number of nodules/plant	D. w. of nodules/plant (g)	D. w. of shoot/plant (g)	% N_2	Total N_2 mg/plant	Nitrogenase activity (µM C_2H_4/dry weight nodules)	Proline accumulation (µM/ g plant)
Non-inoculated	0	0	3.1^{a}	0.3^{a}	0.93^{a}	Not detected	150^{a}
25% N	0	0	6.8^{b}	0.6^{b}	4.1^{b}	Not detected	250^{b}
100% N	0	0	13.3^{c}	1.1^{c}	14.6^{c}	Not detected	530^{c}
Inoculated E15	103^{a}	3.8^{a}	12.2^{cd}	1.9^{d}	23.18^{d}	19.1^{a}	$^{d}910$
Inoculated E15 + 25% N	120^{b}	4.1^{b}	14.2^{ce}	2.3^{e}	32.7^{e}	25.2^{b}	1250^{e}

Means are value of three replications; Means followed by the same letter in a column are not significantly different but by different letters are significantly different (P= 0.05) using the Duncan multiple range test.

conditions with concentration 200 mM NaCl increased the symbiotic N_2-fixation parameters compared with non-inoculated plants (control). The highest values of symbiotic N_2-fixing parameters (no. and dry weight of nodules, dry weight of shoots,% N and total N_2 mg/plant as well as nitrogenase activity) of faba bean plants under field conditions obtained by recombination between inoculation with salt-tolerant rhizobial strain and 25% N fertilizer. These findings are in agreement with Ahmed et al. (1997). The non-inoculated plants were free from nodules, indicating the necessity of using the effective salinity tolerant *Rhizobium* isolate to achieve a good nodulation under salinity and drought stress to improve growth characters of faba bean plants. Data concerning growth parameters are listed in Table 5 and Figure 7 which showed highest values of nitrogenase activity and proline content recorded in inoculated plants with *R. leguminosarum* bv. *viciae* compared with non-inoculated. The highest values of proline accumulation in faba bean plants under field conditions was obtained by recombine-tion between inoculation with salt-tolerant rhizobial strain and 25% N fertilizer and followed by the other treatments. *R. leguminosarum* bv. *viciae* showed significant increase in nodulation parameters compared with uninoculated plants. *R. leguminosarum* bv. *viciae* strain induced significant increase in nodule dry weight and nodule number compared with uninoculated plants grown in saline soil. *R. leguminosarum* bv. *viciae* induced significant increase in root dry weight, shoot dry weight at 90 days from plant sowing compared with uninoculated plants. This indicated that salt tolerant *R. leguminosarum* bv. *viciae* strain reduced the effect of salinity on the N_2 fixation parameters. The results indicated that biofertilization could reduced the severe effects of salinity. From the present work, it could be established that, it's very important to inoculate the saline soil with the salinity tolerant rhizobial isolate. It could be also observed that, efficiency of the salinity tolerant rhizobial isolate was also reduced when faba bean plants were grown in the presence of an efficient *Rhizobium*-legume symbiosis under salt stress and that requires also the selection of salt-tolerant rhizobia. These data are consistent with the results obtained by Graham and Parker (1964). Amarger et al. (1997) noted that tolerance to salinity, acidity, and alkalinity was more strain-specific than species-specific. Similar results have been reported by Nogales et al. (2002).

In agriculture, leguminous biological nitrogen fixation is used to improve infertile soils, especially those affected by salinity (Brockwell et al., 1995). Optimization of the benefits of legume inoculation with rhizobia depends on the survival of rhizobia in soil. The introduction and persistence ability of a strain are affected by a number of biotic factors like high salt, high water potential, high pH, and high temperature (Surange et al. 1997). Therefore, tolerance to high salt and water stresses may be important in the survival, multiplication, and spread of *R. leguminosarum* bv. *viciae* isolated from the root nodules of legumes growing under such stressed conditions is likely when the physiology of these organisms has been carefully studied under these suboptimal conditions. Such rhizobial strains could be used on stressed sites as an inoculum to promote leguminous plant growth in the tropics and sub tropics. Soil degradation due to Stalinization or drought is one of the most serious problems affecting the fertility of soils, especially in arid and semi-arid areas.

Conclusion

Salt and drought- stress are the major constraints to faba bean plants productivity in new reclamation land and isolation of effective rhizobia to inoculate the faba bean plants could be an important strategy to improve the efficiency of rhizobium-faba bean symbiosis and thereby productivity. The results from this study showed that the *R. leguminosarum* bv. *viciae* isolated from different geographic regions soils are able to survive, grow and effectively nodulate faba bean even at high salt concentrations. *R. leguminosarum* bv. *viciae* improved the growth parameters dry weight of shoot as well as total

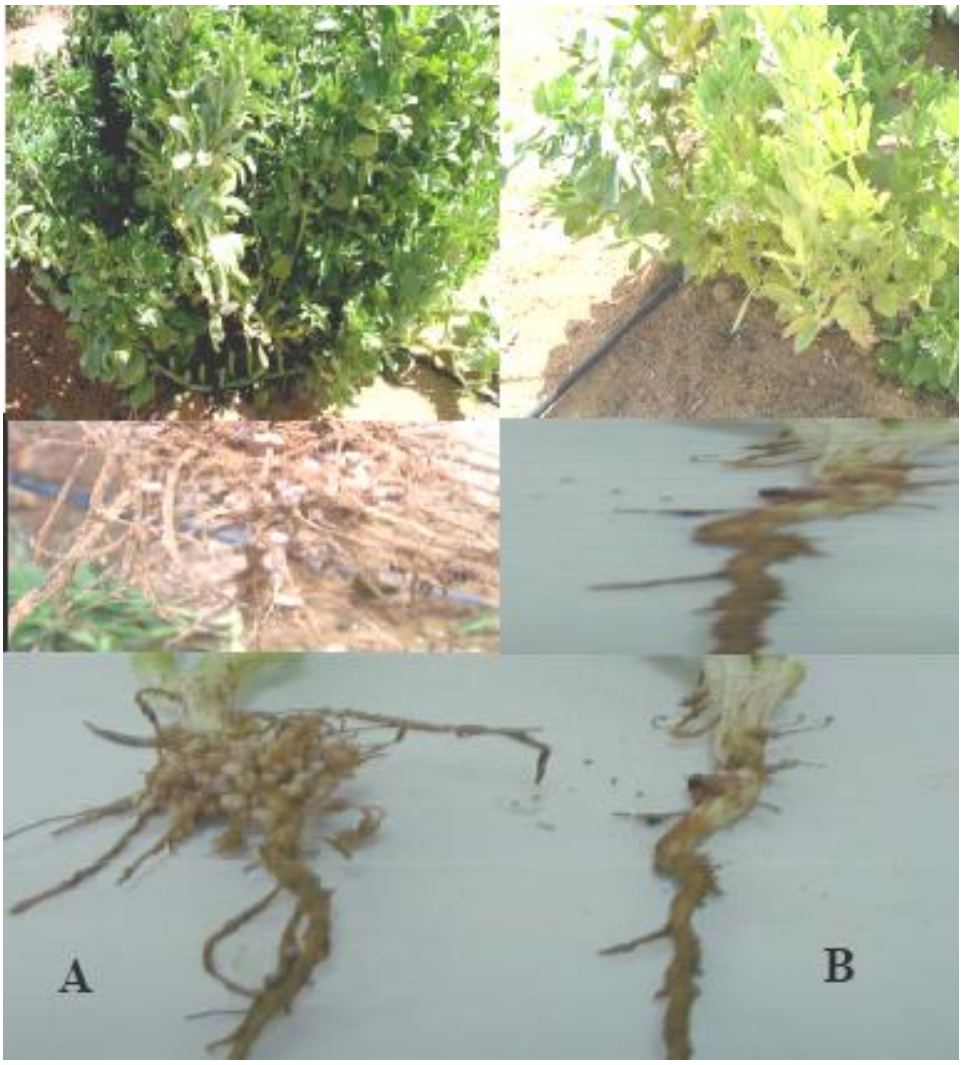

Figure 7. Root systems of non-inoculated and inoculated faba bean Nobaria 1 plants with salinity tolerant isolate of *Rhizobium leguminosarum* bv. *viciae* after 90 days seeds from sowing under field conditions. A, Inoculated; B- non-inoculated.

N_2 mg/plant and proline content of faba bean plants. This strain possesses different characteristics such as tolerance for a biotic stress (salinity and drought), IAA production, phosphate solubilization growth at wide range from pH which can make it candidate for multipurpose inoculants production for faba bean production system in new reclamation land in Egypt.

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