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Full Length Research Paper

Germination, growth and nodulation of *Trigonella foenum graecum* (Fenu Greek) under salt stress

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In this work, we analyzed the effects of salinity on seed germination, growth and nodulation of fenugreek plants. The germination of fenugreek seeds was not affected by salt concentrations lower than 140 mM. Two saline tolerant indigenous rhizobia strains were isolated from the root nodules of fenugreek grown in two different soils. The two rhizobial strains were tested for their ability to grow under high salt stress and then assessed for their ability to nodulate fenugreek grown under different salt stress levels. We have found that the two strains have differential sensitivity to high salt levels. The inoculation of fenugreek with these strains results in better plant growth under salt stress than uninoculated plants. Twenty percent of plants inoculated with strains S9D or S3G survived in 175 mM NaCl, whereas all controls supplemented or not with nitrogen did not grow. Although plants survived in these salt concentrations, many phenotypic alterations were observed, such as stems short length, small number of leaves and reduced fresh weight. No fenugreek plant grew at salt concentrations higher than 175 mM NaCl. Stem and root proteins content was also affected by salinity. However, plants inoculated with the two rhizobial strains were more tolerant to salt than controls. The strains infectivity as estimated by the plants nodules number was also reduced by salinity.

Key words: Fenugreek, plant growth, rhizobia, salt stress, seed germination, nodulation.

INTRODUCTION

Salinity is one of the main factors responsible for soil deterioration and poor agricultural productivity. In semi-arid and arid regions, the extended periods of dryness as well as inappropriate intense irrigation engender a concentration of solutes in soil so that 15% of soils in these regions endure problems of salinity and a third of irrigated lands in the world are affected by the salinity (Hoffman et al., 1980; Jefferies, 1981).

When mineral elements become abnormally concentrated in soils, plants and micro-organisms are submitted to many damages. Saline soils constitute an unfavourable environment for the plant growth. The presence of excess ions produces irreversible biochemical and physiological perturbations in plants; however, some leguminous plant genotypes are naturally tolerant to salinity (Maranon et al., 1989; Niknam and McComb, 2000). Similarly, some rhizobial strains survive in the saline soils (ElSheikh and Wood, 1989, 1990a; Ishaq et al., 1989; Zahran, 1999), although the majority of rhizobia are not capable of tolerating the harmful effects of high osmolarity (Soussi et al., 2001).

The ability of rhizobia to tolerate salt stress depends also on the species and even the strain of rhizobia studied (Bernard et al., 1986). Fast growing rhizobia are generally considered to be more tolerant to saline stress than bradyrhizobia (ElSheikh and Wood, 1990b) and strains isolated from saline soils are typically more tolerant (Hua et al., 1982). Nevertheless, many fast growing rhizobia are very salt sensitive and some rhizobia isolated from saline soils are sensitive (Zahran, 1999).

Rhizobia are known to be more salt tolerant than their plant partners. Maximal limit of tolerance to salinity is superior in rhizobia as compared to their host plant which frequently constitute the limiting factor in saline soils (Kassem et al., 1985).

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Osmotolerant rhizobia use a variety of accumulated or non-accumulated osmoprotectants, including betaines, amino acids and sugars as a strategy to counter the high oscillations of their environment osmolarity (Bernard et al., 1986; Gouffi et al., 1999).

Theoretically, osmotolerant rhizobia would be good candidates for inoculation in zones presenting problems of salinity; they survive and persist in such soils (Dudeja and Khurma, 1989). However, the establishment of nitrogen fixing symbiosis in semi-arid zones depends not only on the selection of efficient bacteria but also in the ability of these bacteria to retain their ability to infect roots in saline soils (Melor et al., 1987).

Fenugreek is one of the old legumes used as a medicinal plant in the Mediterranean region. Actually, it is being widely cultivated in many countries. However, this region is subject to desertification and soil salinity as a consequence to low and random precipitations as well as wrong irrigation practices. Morocco imports all his fenugreek needs from many countries and many farmers in the oriental region of Morocco are interested in its cultivation. As the major area of the eastern Morocco is semi arid to arid and is subjected to desertification and soil salinization, we were interested in the analysis of the effect of salt on the growth of this plant.

The objectives of this work were to assess the effect of salt on: (1) seed germination, (2) plant survival and morphology, (3) and the ability of the plant and rhizobia to establish an effective symbiosis.

MATERIALS AND METHODS

Strains and cultures

The two rhizobia strains tested, S3G and S9D were isolated from root nodules of *Trigonella foenum graecum* (fenugreek) grown in two different soils collected from two fields in the semi-arid to arid regions of the north-east Morocco where no inoculation with any rhizobia had ever been done (Table 1). These two strains are available in Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Laboratoire commun IRD/INRA/CIRAD, Campus de Baillarguet, Montpellier, France.

Fenugreek seedlings were used as hosts to trap rhizobia from many soils of the eastern region of Morocco. Rhizobia nodulating *Trigonella* species are considered to belong to *Sinorhizobium* genus (Allen et al., 1981). The two isolates were identified by rep PCR as *Sinorhizobium* sp. (results not shown).

Each isolate was recovered by the standard method of Vincent (1970) on YEM medium and maintained on YEM agar slants. Purity was controlled regularly by repeated streaking.

Seeds sampling and germination

Seeds of *T. foenum graecum* are sold (to medicinal and culinary purposes) in the markets of Oujda (Morocco). Seeds were surface sterilized by 5 min exposure to 3% (w/v) calcium hypochlorite, thoroughly rinsed with sterile distilled water and transferred to plates containing water agar (6% w/v) and then incubated in the dark at 25°C to promote germination. 50 seeds were used in each test with two repetitions.

Tests of nodulation

All isolates were tested for nodulation of their original host. Seedlings were grown in autoclaved jars (10 cm^2 (S) x 20 cm (H)) containing a dilution of Jensen nitrogen-free solution (Vincent, 1970). Jars were placed in a growth chamber at 22°C, with illumination for 14h/day. Strains were grown on YEM slants for 48 h and the colonies obtained were suspended in 3 ml bi-distilled sterile water. A volume of 2 ml of the suspension containing 10^8 ¢/ml was aseptically inoculated onto seedling roots. Nodulation was checked after a period of two to four weeks.

Effect of salt on the growth of the two strains

The effect of NaCl concentrations on the growth of the two strains was assessed in a mineral liquid broth after 6 days incubation period, by determining the absorbance at 600 nm with a spectronic 20 D spectrophotometer (Abdelmoumen et al., 1999). The salt was added to the medium before autoclaving to give the concentrations: 170, 350, 510, 700, 875 mM: 1.05, 1.225, 1.4, 1.57 and 1.75 M. Media were dispensed as 5 ml aliquots into test tubes and inoculated after autoclaving with a 36 h culture (10^8 ¢/ml) of the appropriate strain in the mineral medium. Cultures were grown at 28°C in a rotary shaker at 70 rpm.

The effect of salt on rhizobia was also tested on YEM agar media containing NaCl concentrations 170, 350, 510, 700, 875 mM, 1.05, 1.225, 1.4, 1.57, 1.75, 2.1 and 2.45 M. Plates were inoculated by streaking from young cultures of about 10^8 cells/ml and incubated at 28°C.

Germination of seeds under saline stress

After disinfection, seeds were placed to germinate on water agar plates containing NaCl concentrations of 35, 70, 105, 140, 175, 210, 245, 280, 315, 350, 385, 420 and 455 mM. Plates were incubated at 26°C in the dark and germination was assessed periodically.

Effect of the NaCl on the infectivity and the efficiency of the two strains

The effect of different NaCl concentrations on the growth of fenugreek plants was assessed in plants inoculated by strains S3G or S9D as well as two non inoculated controls supplemented or not with nitrogen (KNO_3). Hence, the seedlings obtained after germination of seeds on water agar plates without any salt were transferred in pots containing sterilized river sand and inoculated by 3 ml of 10^8 ¢/ml culture suspensions of strains S3G or S9D. They were watered twice a week with a dilution of Jensen nitrogen-free solution containing different NaCl concentrations; 17, 35, 51, 70, 105, 140 and 175 mM. Controls were watered by the same nutritive solutions containing (positive controls) or not (negative controls)) 0, 5 g/l of KNO_3.

The growth was estimated by measuring the stem size, the number of leaves and the total plant fresh weight. With inoculated plants, we determined the effect of salt on the nitrogen fixation and infectivity of the two strains. The nitrogen fixation was estimated by the total plant proteins in comparison with controls. We considered that there is a strong correlation between the nitrogen fixed and total protein content of the plant since the majority of the fixed nitrogen will be found in amino acids that will constitute the cellular proteins. Infectivity or the aptitude of the strain to produce nodules on plant roots was estimated by the number and weight of nodules obtained on the plant roots.

Table 1. Provenance of soils used to trap rhizobia that nodulate fenugreek.

Soil sampling site	Strain	Type of substrate	Bio climatic Status
Lalla Mimouna	S9D	Calcareous marls	arid with cool winter 0°C < m < 3°C
Debdou	S3G	Schist and granite	Semi-arid with cold winter m < 0°C

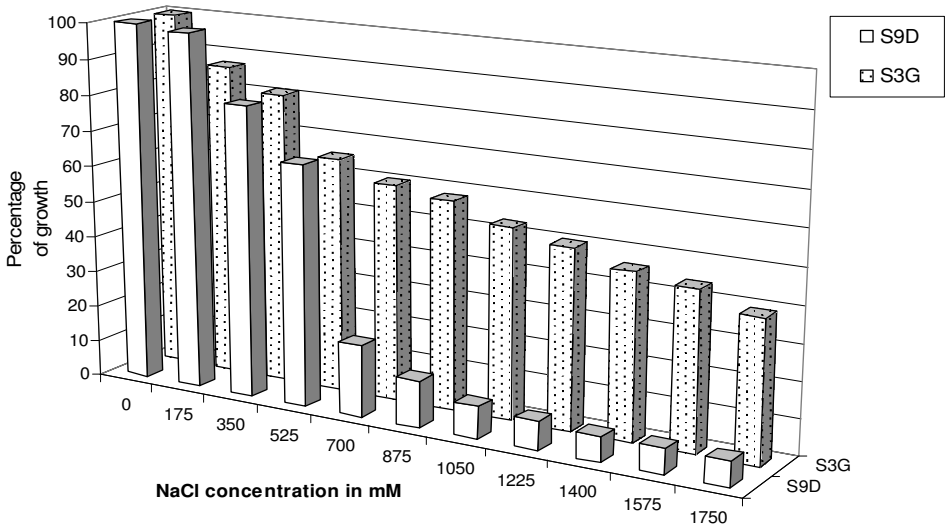

Figure 1. Effect of salt on the growth of strains S3G and S9D. Percentage of growth defined against a control containing no salt incubated in the same conditions.

We determined also the percentage of plant survival after five weeks in salt stressed media. Five plants were used in each test and all tests were repeated in twice.

RESULTS

Effect of salt on the growth of strains

Strain S3G was more tolerant to high salt concentrations than S9D (Figure 1). As the salt concentration increased, growth inhibition of the strains also increased. In 1.225 M of NaCl, the inhibition of S9D was drastic and reached 90%; while in S3G, the inhibition of the growth was approximately 50%. Strain S3G continued to grow in 2.1 M NaCl but was completely inhibited by 2.45 M of NaCl (data not shown).

Effect of salt on seeds germination

The germination of fenugreek seeds was not affected by salt concentrations lower than 140 mM (Figure 2). In this salt concentration, we observed a slight delay in the time necessary for the germination of seeds. With 175 mM of NaCl, 100% of the seeds germinated after five days, whereas with 210 mM, 100% of the seeds germinated after ten days. In higher concentrations, this percentage was no longer obtained and the number of germinated seeds decreased with the NaCl concentration increase. Only 50% of seeds germinated after 15 days in 350 mM NaCl.

Effect of salt stress on plant growth

The effect of high salt concentrations damaged the growth of inoculated as well as not inoculated plants. Hence, controls without nitrogen showed no growth at NaCl concentrations higher than 105 mM. After five weeks, the percentage of surviving of non inoculated plant controls supplemented with KNO_3, decreased to 60% in presence of 70 mM NaCl (Figure 3).

The inoculated plants were relatively less affected than the controls. The percentage of survival was 100% in 35 mM of NaCl. In 70 mM NaCl, it was 80% in plants inoculated with S9D and 60% in plants inoculated with S3G. This percentage decreased and reached 60% with S9D and 40% with S3G in 140 mM NaCl. Contrarily to controls, 20% of the plants inoculated with S9D as well as with S3G survived at 175 mM of NaCl and no plant supported concentrations higher than 175 mM. However, even if plants survived in these salt concentrations, many alterations were observed, such as a reduction in the stems length and leaves number as well as a decrease in the total plant fresh weight. These alterations were more

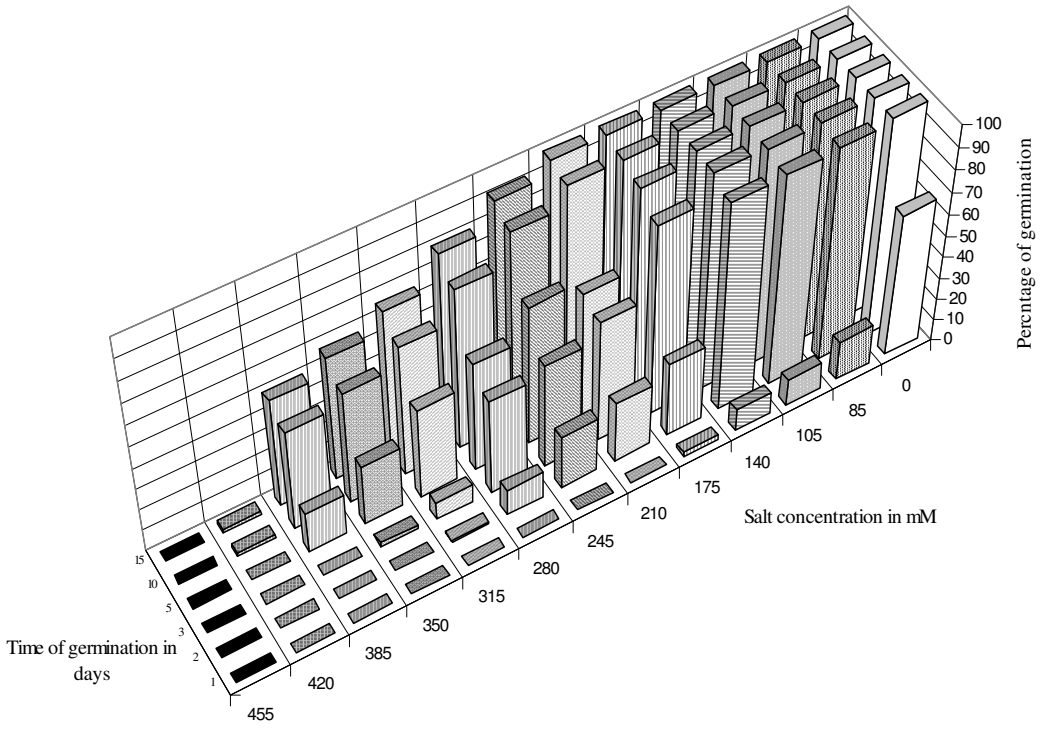

Figure 2. Effect of salt concentration on seed germination. Germination was assessed after 1, 2, 3, 5, 10 and 15 days incubation in darkness at 25°C.

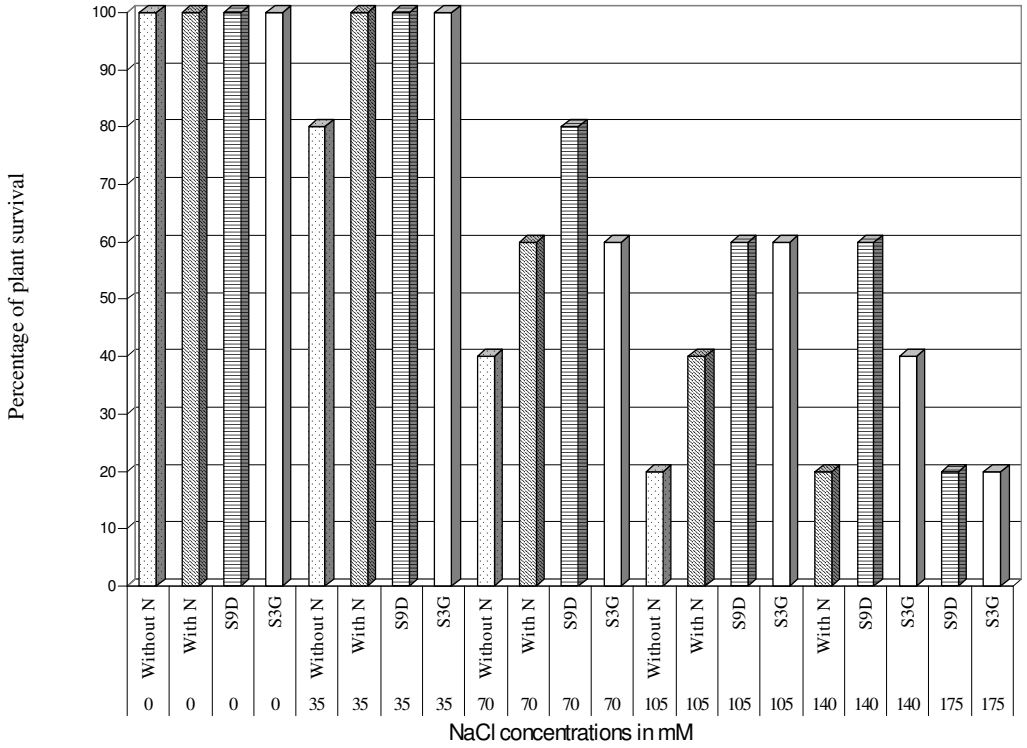

Figure 3. Percentage of survival of inoculated fenugreek plants grown in presence of different salt concentrations after five weeks. Controls containing nitrogen (as KNO_3) or not were assessed in the same conditions.

Table 2. Effect of salt concentration on plants inoculated or not with strains S9D and S3G.

Salt concentration (in mM)	Assay	Stem length (In cm)	Leaves number per plant	Fresh weight (in g)
0	- N_2	9 ± 0,8	12 ± 3	3.1 ± 0.5
	+ N_2	12 ± 1.2	14 ± 2	6.4 ± 1.3
	S9D	10 ± 0.7	15 ± 3	5.3 ± 1.2
	S3G	11 ± 0.9	13 ± 2	4.5 ± 0.7
35	- N_2	7.3 ± 0.6	8 ± 2	4.2 ± 1
	+ N_2	8 ± 0.4	8 ± 1,5	5.1 ± 0.2
	S9D	8 ± 0.6	12 ± 3	5.8 ± 0.6
	S3G	8 ± 1.3	11 ± 3	5.6 ± 0.45
70	- N_2	4.5 ± 0.3	6 ± 2	1.3 ± 0.2
	+ N_2	5 ± 0.7	6 ± 2	2 ± 0.2
	S9D	6 ± 1	9 ± 2	3.6 ± 0.3
	S3G	6.2 ± 0.8	9 ± 2	3.9 ± 0.4
105	- N_2	3 ± 0.3	5 ± 1.5	0.5 ± 0.1
	+ N_2	3.4 ± 0.2	5 ± 1	0.7 ± 0.2
	S9D	4,3 ± 0.5	6 ± 0.5	2 ± 0.3
	S3G	5,1 ± 0.4	7 ± 0.35	3 ± 0.3
140	+ N_2	2 ± 0.3	2 ± 0.4	0.3 *
	S9D	3.9 ± 0.3	6 ± 1	1.5 ± 0.3
	S3G	4.2 ± 0.2	6 ± 0.8	1.2 ± 0.2
175	S9D	3.3 ± 0.4	6 ± 1.2	0.8 ± 0.12
	S3G	2,2 ± 0.6	2 ± 0.2	0.4 ± 0.1

The effect of salt was tested with plants inoculated with strains S9D or S3G and controls supplemented (+ N_2) or not (- N_2) with nitrogen (as KNO_3). Each value is a mean of two tests.
*Only two plants survived in all the repetitions.

important in controls than in inoculated plants (Table 2). In 35 mM of NaCl, there was a decrease of approximately 20% of stems size and leaves number of controls, whereas the fresh weight was not affected. With the same concentration, this reduction exceeds 30% for all parts of the plant in nitrogen supplemented controls. In 175 mM of NaCl, damages were more important in plants inoculated with the strain S3G than plants inoculated with S9D. The reduction of control plants stem size, leaves number and fresh weight exceeded 90% in presence of 140 mM of salt; whereas no growth was observed in 175 mM NaCl.

Effect of salt concentration on symbiosis

There was a reduction in root and shoot proteins correlated with the augmentation of salt concentration of the irrigation solution (Figure 4). The reduction of total protein content was approximately 20% in 35 mM of NaCl and it continued to decrease and reached 80% in 140 mM of NaCl, whereas in plants inoculated with S9D, this diminution was of 10, 50 and 64% in presence of NaCl concentrations 35, 70 and 175 Mm, respectively. Plants inoculated with S3G showed a total proteins reduction of 20, 50 and 70%, respectively. However, the effect of salt was more harmful on nitrogen supplemented controls.

The number and the weight of root nodules permitted the estimation of the infectivity of the strains. The ability of strains to nodulate their host plant diminished rapidly with the increase of NaCl concentration (Figure 5). The reduction of nodule number was more important with strain S3G that S9D, although strain S3G is more tolerant to salinity than S9D. In 35 mM of NaCl, the number of nodules decreased to approximately 50% in plants inoculated with S3G, while in plants inoculated with S9D there was a diminution of 20%. The number and weight of nodules continued to decrease with the increase of salt concentration to reach 82 and 79%, respectively, in plants inoculated with S9D, and 87 and 80%, respectively, in plants inoculated with S3G at 140 mM of NaCl.

DISCUSSION

The two strains isolated from fenugreek root nodules are salt tolerant. Strain S9E still grows at a concentration of 1.05 M of NaCl and strain S3G continues to grow at a concentration of 2.1 M. Such level of tolerance has only been described previously by Zahran et al. (1994) who

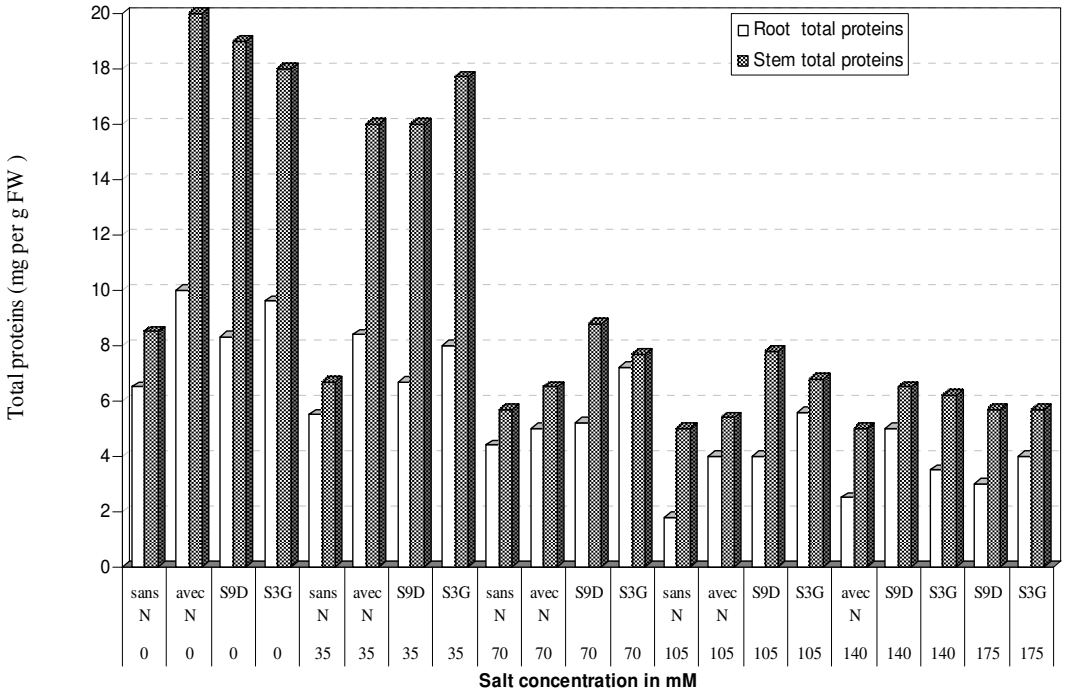

Figure 4. Effect of salt on root and stem total soluble proteins. Units are expressed as total protein quantity in mg by g of plant fresh weight.

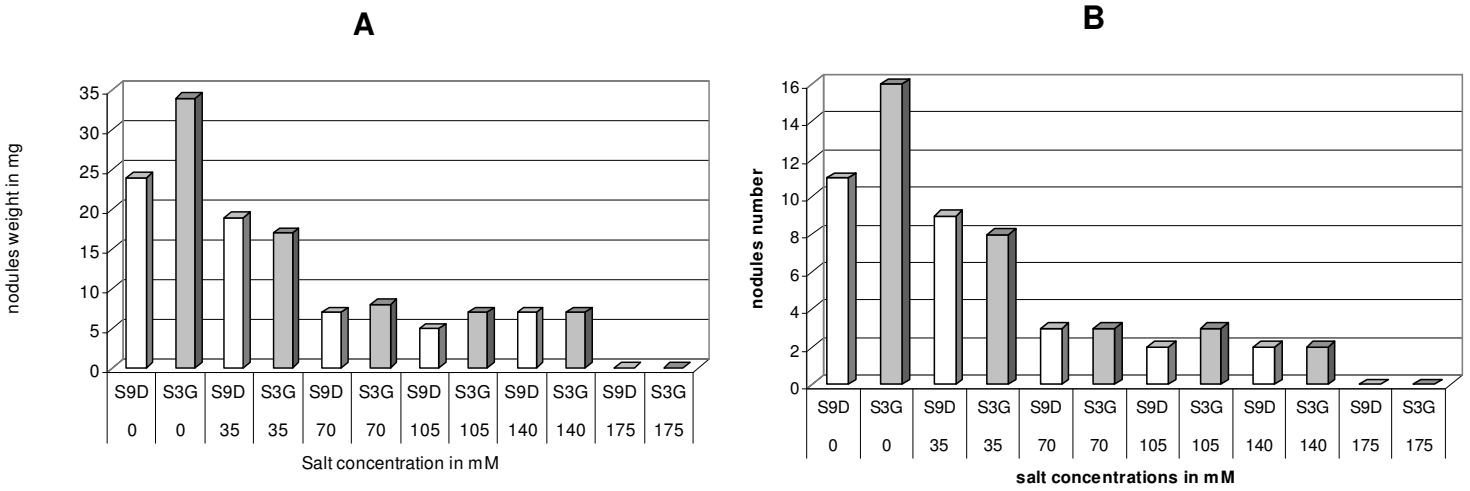

Figure 5. Effect of salt on nodule weight (A) and nodule number per plant (B) inoculated with strains S3G or S9D.

isolated some rhizobia capable of growing on culture media containing 1.75 M of NaCl. However, although strain S3G is more tolerant to high salt concentrations than S9D, the growth of S9D is superior at concentrations below 700 mM to strain S3G.

To survive in salt stressed conditions, bacteria develop many mechanisms that allow them to keep their vital metabolic activities in function, such as the accumulation of potassium ions, glutamate, sugars and many compatible solutes such as betaines and ectoines (Le Rudulier and Bernard, 1986; Le Rudulier, 1993; Jebbar et al., 1992; Talibart et al., 1994; Gouffi et al., 1998, 1999, 2000). We have already shown that these two strains may use glycine betaine, proline and glutamate asosmoprotectants in high salt concentrations (Abdelmoumen et al., 1999).

Fenugreek seeds germination was not inhibited by concentrations that inhibit the plant growth. They produce trigonelline ($C_7H_7NO_2$) which is a betaïne (N - methylbetaine) of nicotinic acid and choline (precursor of the glycine-betaine) (Allen and Allen, 1981). Betaines are known for their osmoprotective effects in micro-organisms

and plants (Le Rudulier, 1993; Phillips et al., 1998; Boncompagni et al., 1999). However, there was no relationship between the ability of this plant to germinate in salt concentrations higher than 175 mM and its growth in these conditions. No control (even nitrogen supplied) developed in concentrations higher than 105 mM, although seeds germination was not so affected by salt and continued until 420 mM NaCl concentration.

Although the two strains of rhizobia were salt tolerant, the symbiosis was affected by salinity, and inoculated plants did not nodulate at salt concentrations higher than 140 mM of NaCl.

As we considered that in legumes, total protein content may be related to symbiotic nitrogen fixation, we determined root and stem total proteins to have an indication on the efficiency of the two strains. We observed that the efficiency of the two strains was also altered by the osmotic stress.

The number and weight of root nodules of fenugreek were significantly affected by NaCl concentrations that did not cause any problem to rhizobial proliferation. However, the inoculation with efficient rhizobia sustains the survival of plants in salt concentrations where even nitrogen supplied controls could not grow. This confirms that the presence of rhizobia or the symbiosis establishment might improve the tolerance of plants in salt stressed environments. Sprent and Zahran (1988) previously showed that the number of nodules decreases strongly in concentrations that do not affect density of rhizobia in soil. Many properties of rhizobia can be altered by salt stress, such as the production of the lipo-polysaccharides (LPS) that play an important role in the interactions between the external membrane of the bacterium and the plant membranes glycoproteins (Carlson et al., 1987; Soussi et al., 2001). It has been also reported that salinity alters plant root hair development (Zahran and Sprent, 1986).
Hence, in order to introduce fenugreek in saline soils of the eastern Morocco, in addition to the selection of bacterial strains resistant to salt stress and preserving their infectivity and efficiency in these conditions, it is necessary to select plant genotypes that are also tolerant to salinity.

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