Symbiosis, 12 (1992) 311–319
Balaban, Philadelphia/Rehovot

Nodulation and Nitrogen Fixation in Faba Bean (*Vicia faba* L.) Plants under Salt Stress

M.H. ABD-ALLA
Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt
Tel. 20 (88) 333555, Telex 92863 ASUNV UN, Fax 20 (88) 323564

Received February 20, 1992; Accepted July 12, 1992

Abstract

Faba bean cultivar Giza 3 inoculated with *Rhizobium leguminosarum* RCR 1001 was grown in a pot experiment and irrigated with saline water (mixture of NaCl and $CaCl_2$ 0.25 Ca:Na on molar basis). Salinity levels of 5.8, 8.8, 11.6 and 14.6 dSm^{-1} (equivalent to 50, 75, 100 and 125 mM NaCl) significantly decreased nodule number, nodule fresh weight and total nitrogenase activity. Salinity inhibited specific nitrogenase activity, protein, leghaemoglobin and carbohydrate content of the nodules at 11.6 and 14.6 dSm^{-1} (100 and 125 mM NaCl). Salinity levels of 8.8, 11.6 and 14.6 dSm^{-1} (75, 100 and 125 mM NaCl) caused a significant reduction in dry weights of roots, stems, leaves and total plant nitrogen. The results indicated that *Rhizobium leguminosarum* RCR 1001 formed an infective and effective symbiosis with faba bean under saline conditions. A noticeable decline in nitrogenase activity and total nitrogen concentration in the plant under saline conditions could be attributed to nodule senescence, as shown by the lowering trend of leghaemoglobin, soluble protein and carbohydrate contents of cytosol and bacteroids.

Keywords: acetylene-reducing (nitrogenase) activity, nodulation, *Rhizobium*, salinity, *Vicia faba*

1. Introduction

Salinity is an osmotic stress responsible for major crop losses the world over, especially in semi-arid soil and irrigated agriculture. The area affected

0334-5114/92 /$03.50

by salinity in Egypt and elsewhere is increasing each year. In legumes salinity can result in a root system devoid of root hairs and a mucilaginous layer, and incapable of forming an infection thread (Singleton and Bohlool, 1984; Zahran and Sprent, 1986).

Unsuccessful symbiosis may be attributed to failure in the infection process due to poor establishment of *Rhizobium* (Rai and Prasad, 1983). The reduction of nodulation in soybean under saline conditions was attributed to shrinkage of the root hairs (Tu, 1981). Yousef and Sprent (1983) showed that NaCl affected nodulation and they concluded that there may also be effects on infection.

There have been only a few studies (Salem et al., 1982; Yousef and Sprent, 1983) on the effect of salinity on growth, nodulation and nodule physiology of *Vicia faba*. In this investigation it was intended to study the effect of a continuous supply of salinity on the nodulation, symbiotic nitrogenase activity and plant growth of *Vicia faba*. Protein, carbohydrate and leghaemoglobin contents of nodules (cytosol and bacteroids) were also assessed.

2. Materials and Methods

Plant culture and experimental conditions

Surface-sterilized faba bean seeds (*Vicia faba* L. cv. Giza 3) were inoculated with *Rhizobium leguminosarum* RCR 1001 (from Rothamsted Experimental Station, Harpenden, UK) and planted into each of 17 cm diameter plastic pots containing 3 kg sterilized clay soil. The soil characteristics of the experimental site are shown in Table 1. Seedlings were thinned to two per pot after 5 days. Plants were grown in a wire proof greenhouse maintained at 24±4°C, under natural day light in October-November.

Five levels of salinity (mixture of NaCl and $CaCl_2$, 0.25 Ca:Na on molar basis) of 0, 5.8, 8.8, 11.6 and 14.6 dSm^{-1} at 28°C (equivalent to 0, 50, 75, 100 and 125 mM NaCl) were used.

Irrigation with saline water started 5 days after planting and 30 ml of relevant solution was given daily to each pot for 25 days. Thereafter the plants received only tap water until the end of the experiment. Plants were harvested 8 weeks after starting the salt treatment (61 days after planting). This time period has provided large enough samples of nodules for analysis.

Analyses

Nitrogenase activity was determined on a detached root system, using the acetylene reduction assay (Hardy et al., 1968). Assays were conducted in a closed system. Roots were cut-off at coteledonary nodes, gently shaken to

Table 1. Some physical and chemical properties of a representative soil sample of the experimental site

Properties	Values
Texture analysis (w/w)	
Clay %	45.6
Silt %	30.25
Sand %	20.60
Textural grade	
Saturation % H_2O	70.0
Field capacity % H_2O	70.4
Ece mmhos/cm (1:2)	1.51
Soluble cations	
Ca^{++}meq/100 g soil	0.29
Mg^{++}meq/100 g soil	0.27
Na^{+}meq/100 g soil	0.55
K^{+}meq/100 g soil	0.04
Soluble anions	
CO_3^{2-} + HCO_3^{-}meq/100 g soil	0.25
Cl^{-}meq/100 g soil	0.75
SO_4^{2-}meq/100 g soil	0.08
pH (1:2 soil:water suspension)	7.5
Organic matter %	1.55
$CaCO_3$ %	5.45
Total nitrogen %	0.05

remove loose soil particles, placed in 556 ml mannitol bottles and sealed with a rubber septum. Fifty-five ml of acetylene were injected into the bottles which were then incubated at 28°C for 1 hr. The reaction was terminated using HCl (6 N). A 500 μl gas sample for each bottle was injected into a Pye Unicame 104 Gas Chromatograph containing a flame ionization detector and a 5 ft×1/8 inch glass column of activated alumina (80–100 mesh) at a temperature of 150°C. The carrier gas was nitrogen at 30 ml/min. Afterwards nodules of each individual root were counted and nodule fresh weights were measured. Dry weights of leaves, stems, roots and total nitrogen content of each organ were determined using Kjeldahal digestion (Black et al., 1965).

Nodule fractionation and bacteroid isolation

One gram of nodules was rinsed thoroughly with distilled water and immediately handground in an ice-chilled mortar with 5 ml of bidistilled water. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 500 g for 2 min to remove nodule debris. The resulting supernatant was centrifuged at 12,000×g for 15 min to sediment

the bacteroids. The supernatant, referred to as nodule cytosol, was used for the determination of protein, leghaemoglobin and carbohydrate. Bacteroids were resuspended in bidistilled water and broken by Sonication (MSE Ultrasonic Disintegrator, MSE Co., USA) for 8 min. Cell debris was removed by centrifugation at 25,000×g for 30 min and the supernatant was saved for protein and carbohydrate determinations. Protein was determined according to Lowry et al. (1951). For the determination of water-soluble carbohydrates, the phenol-sulphuric acid method was used (Dubois et al., 1956). Leghaemoglobin was measured by colorimetry, essentially as described by Larue and Child (1979) using spectronic 2000 (Bausch and Lomb). The colorimetric assay was standardized using freshly prepared Hemetrol reagent (solution of cyanmethemoglobin titrated exactly according to the recommendations of BioMerieux, Marcyletoile, 69260 Chabon nieres les Bains, France).

3. Results and Discussion

Nodulation

The total number of nodules on faba bean cv. Giza 3 plants significantly reduced as salinity increased. The inhibitory effect of salt on nodulation of *Vicia faba* appeared at the lowest salt level of 5.8 dSm^{-1} (50 mM NaCl) and became more inhibitory with increase in salt concentration (Table 2). The reduction in number was 24, 40, 51 and 68% for 5.8, 8.8, 11.6 and 14.6 dSm^{-1}, respectively.

Table 2. Effect of salinity ($NaCl + CaCl_2$) on nodulation and nodule activity of *Vicia faba* cv. Giza 3 inoculated with *Rhizobium leguminosarum* RCR 1001. Each value represents the mean of three replicates.

Salt level dSm^{-1}	Nodule no. (per plant)	Nodule F.W. (mg/plant)	Acelytne reduction	
			μmol C_2H_4/hr/plant	n mol C_2H_4/hr/mg nodule F.W.
0	126.7	425	3.63	8.90
5.8	96.0	355	3.02	8.63
8.8	75.7	250	2.08	8.43
11.6	61.0	209	1.19	5.20
14.6	40.0	116	0.65	4.73
L.S.D				
P = 5%	3.4	11	0.10	1.29
P = 1%	5.0	16	0.15	1.38

In salt-treated plants, total nodule weight per plant was reduced with increasing amounts of salt given. A reduction of 16.7, 41.2, 50.8 and 72.6% in nodule weight was caused by salinity levels of 5.8, 8.8, 11.6 and 14.6 dSm^{-1}, respectively. Total nodule weight per plant followed a similar pattern to nodule number per plant.

These results agree with the results of Yousef and Sprent (1983), Zahran and Sprent (1986), and Elsheikh and Wood (1990). Twenty mM NaCl (2.3 dSm^{-1}) inhibited nodulation of ckickpea inoculated with four different *Rhizobium* strains by 16–20% (Lauter et al., 1981). Hafeez et al. (1988) reported that the nodulation of *Vigna radiata* was reduced by about half at a salinity level of 5.0 dSm^{-1} compared to 1.4 dSm^{-1}. They also found that nodulation was completely depressed when salinity was raised to 10.0 dSm^{-1}, regardless of the plant growth stage. In the experiments reported here nodulation was recorded at 125 mM NaCl (14.6 dSm^{-1}). The reduction of failure in nodulation at high salinity might be attributed to shrinkage of root hairs (Tu, 1981; Zahran and Sprent, 1986). The processes of nodule initiation in soybean was reported to be extremely sensitive to NaCl. A reduction in nodule number and weight of 50% occurred with 26.6 mM NaCl (3.1 dSm^{-1}) in the rooting medium (Singleton and Bohlool, 1984). In this study, nodulation was reduced by 24% at 50 mM NaCl (5.8 dSm^{-1}).

Nodule activity

Nitrogenase (acetylene-reduction) activity per plant (total activity) was severely depressed by salinity (Table 2). A reduction of 16.8, 42.2, 67.2 and 82.1% in total activity was caused by salinity levels of 5.8, 8.8, 11.6, 14.6 dSm^{-1}, respectively. The results suggest that the depression in total activity was due to salt reducing the nodule fresh weights per plant (Table 2). Specific nitrogenase activity (ethylene production per mg nodule) was significantly inhibited by salinity levels of 11.6 and 14.6 dSm^{-1} (100 and 125 mM NaCl) (Table 2).

In salt-treated nodules, leghaemoglobin content was reduced with rising amount of salt. A reduction of 17, 13, 14 and 53% in leghaemoglobin content was caused by salinity levels of 5.8, 8.8, 11.6 and 14.6 dSm^{-1}. The reduction in protein content of the bacteroids was 1.4, 10.4, 11.5 and 43.5 and of the corresponding cytosol was 11.9, 22.7, 25.6 and 60% at salinity levels of 5.8, 8.8, 11.6 and 14.6 dSm^{-1}, respectively. Also, carbohydrate contents of both the bacteroids and the cytosol were decreased by 1.7, 7.1, 12.5 and 32.2 and 21, 39, 45 and 70% at salinity levels of 5.8, 8.8, 11.6 and 14.6 dSm^{-1}, respectively (Table 3).

Table 3. Effect of salinity on protein, carbohydrate and leghaemoglobin (Lhb) content of *Vicia faba* nodules. Each value represents the mean of three replicates.

Salt level dSm^{-1}	Lhb (mg(g fw nodules)$^{-1}$)	Protein concentration (mg(g fw nodules)$^{-1}$)		Carbohydrate (mg(g fw nodules)$^{-1}$)	
		Bacteroid	Cytosol	Bacteroid	Cytosol
0	2.15	3.65	4.53	0.56	1.66
5.8	1.78	3.60	3.99	0.55	1.31
8.8	1.80	3.27	3.50	0.52	1.01
11.6	1.25	3.23	3.37	0.49	0.91
14.6	1.01	2.06	1.79	0.43	0.49
L.S.D.					
P = 5%	0.48	0.11	0.34	0.05	0.08
P = 1%	0.70	0.16	0.49	6.08	0.11

It is likely that the depression in specific nitrogenase activity was due to salt reducing the protein, leghaemoglobin and carbohydrate contents of both the cytosol and the bacteroids (Table 3). Salinity levels at 11.6 and 14.6 dSm^{-1} induced a 37 and 59% decline in carbohydrate and 41 and 53% in leghaemoglobin and 19 and 53% in protein content of nodules, while nodule C_2H_2 reduction (specific nitrogenase activity) showed a 55 and 61% decreases, respectively. Protein synthesis is readily inhibited by water stress because of the decreases in the level of polyribosomes (Bewley, 1981). Thus, the decline of nodule soluble protein may result from a general reduction of protein synthesis and from an increased protease activity in the cytosol (Becana et al., 1986). The disappearance of nodule soluble protein induced by plant water stress can be compared to the situation naturally occurring during nodule senescence (Pladys and Rigaud, 1985; Vance et al., 1986) or after feeding the plant with nitrate (Pladys et al., 1988). In both cases, the presence of active proteases was responsible for the protein digestion, and it seems likely that these enzymes may also be induced under water restricted conditions (Guerin et al., 1990; 1991).

The results presented in Table 3 suggest that most biochemical parameters of the bacterial and plant components of the nodules (cytosol) were affected by high levels of salinity (100 and 125 mM NaCl). Both the protein and carbohydrate contents of nodule cytosol were more sensitive to salinity than the corresponding bacteroids at the same levels of salinity.

Plant growth and nitrogen content

An inhibitory effect of salt on the dry weight of the root, stem and leaves was apparent at salinity levels of 8.8 dSm^{-1} (75 mM NaCl). Salinity levels of 8.8, 11.6 and 14.6 dSm^{-1} caused a reduction in dry weight of each organ (Table 4). Also, salinity significantly decreased the total nitrogen of each organ

Table 4. Effect of salinity on dry weights and total nitrogen of plants. Each value represents the mean of three replicates.

Salt value dSm^{-1}	Dry weight (mg/plant)			Total nitrogen* (mg/plant)		
	Root	Stem	Leaves	Root	Stem	Leaves
0	256	205	321	21.20	23.53	30.30
5.8	261	205	318	20.66	22.03	29.93
8.8	245	192	287	18.56	19.70	29.03
11.6	195	177	221	8.30	10.20	13.66
14.6	173	107	153	6.17	8.03	11.86
L.S.D.						
P = 5%	8.9	6.6	11.0	1.53	1.88	3.36
P = 1%	12.9	9.6	15.9	2.18	2.68	4.78

* Sum of nodule N_2 fixation and N uptake from soil

(Table 4).

Salinity has been reported to reduce shoot and root weights in legumes, e.g. faba bean (Yousef and Sprent, 1983; Zahran and Sprent, 1986), soybean (Grattan and Mass, 1988) and chickpea (Lauter and Munns, 1987). For *Vigna radiata*, at maturity (65 d), 95% of the plants survived at 7.5 dSm^{-1} (68 mM NaCl). However, all plants died at 10.0 dSm^{-1} (90.0 mM NaCl) regardless of the presence or absence of inoculation (Hafeez et al., 1988). Results reported here confirm that faba bean cv. Giza 3 is less sensitive to saline conditions, compared to other legumes reported above.

The sensitivity of plants to salt may be linked to the inhibition of plant growth through accumulation of Na in the shoots due to the inability of the plant to decrease transpiration (Lauter and Munns, 1986). Excessive accumulation of Na adversely affects enzymatic activity, photosynthesis rate, and the translocation and further utilization of photosynthates (Poljakoff-Mayber, 1982; Durand et al., 1987; Sheoran et al., 1988; Vassey and Sharkey, 1989). The reduction in plant growth under saline conditions could be accounted for in part by the depression in nitrogenase activity. This was closely related to the decrease in the content of carbohydrate in the nodules, due to decrease in photosynthesis (Table 3).

In conclusion, decreased N_2-fixation in faba bean nodules under salt stress seems to some extent to be due to nodule senescence, as shown by the lowering trends of acetylene reduction activity, leghaemoglobin and soluble protein and carbohydrate contents of both the cytosol and the bacteroid fractions. More

work is required to elucidate the mechanisms by which salinity interferes with normal nodule physiology and function.

REFERENCES

Abdel-Ghafar, A.S., EL-Attar, H.A., EL-Halfawi, M.H., and Abdel-Salam, A.A. 1982. Effects of inoculation, nitrogen fertilizer, salinity and water stress on symbiotic N_2 fixation by *Vicia faba* and *Phaseolus vulgaris*. In: *Biological Nitrogen Fixation Technology for Tropical Agriculture*. P.H. Graham and S.C. harris, eds. Centro Int. de Agricultura Tropical, Cali Colombia, pp. 153–159.

Abdel-Wahab, A.M. and Zahran, H.H. 1981. Effect of salt stress on nitrogenase activity and growth of four legumes. *Biologia Plantarum* (praha) **23:** 16–23.

Becana, M., Aparicio-Tejo, P.M., and Sanchez-Diaz, M. 1986. Nitrate metabolism of alfalfa root nodules under water stress. *J. Expt. Bot.* **37:** 353–358.

Bewley, J.B. 1981. Protein synthesis. In: *The Physiology and Biochemistry of Drought Resistance in Plants*. L.G. Paleg and D. Aspinall, eds. Academic Press, New York, pp. 261–282.

Black, C.A., Evans, D.D. and Emsminger, L.E. 1965. *Methods of Soil Analysis*. Agronomy, 9. Amer. Soc. Agron., Inc., Madison, WS. pp. 1171–1174.

Dubois, M., Gillies, K.A., Hamilton, J.A., Rebers, P.A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substance. *Anal. Chem.* **28:** 350–356.

Durand, J.L., Sheehy, J.E., and Minchin, F.R. 1987. Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water deprivation. *J. Exp. Bot.* **38:** 311–321.

Elsheikh, E.A.E. and Wood, M. 1990. Effect of salinity on growth, nodulation and nitrogen yield of chickpea (*Cicer arietinum* L.) *J. Expt. Bot.* **41:** 1263–1269.

Grattan, S.R. and Mass, E.V. 1988. Effect of salinity on phosphate accumulation and injury in soybean: I. Influence of $CaCl_2$/NaCl ratios. *Plant Soil* **105:** 25–32.

Guerin, V., Trinchant, J., and Rigaud, J. 1990. Nitrogen fixation (C_2H_2 reduction) by broad bean (*Vicia faba* L.) nodules and bacteroids under water-restricted conditions. *Plant Physiol.* **92:** 585–601.

Guerin, V., Pladys, D., Trinchant, J., and Rigaud, J. 1991. Proteolysis and nitrogen fixation in faba-bean (*Vicia faba*) nodules under water stress. *Physiol. Plant.* **82:** 360–366.

Hafeez, F.Y., Aslam, Z., and Malik, K.A. 1988. Effect of salinity and inoculation on growth, nitrogen fixation and nutrient uptake of *Vigna radiata* (L.) Wilczek. *Plant Soil* **106:** 3–8.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., and Burns, R.C. 1968. The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. *Plant Physiol.* **43:** 1185–1207.

Larue, T.A. and Child, J.J. 1979. Sensitive fluorometric assay for leghaemoglobin. *Anal. Biochem.* **92:** 11–15.

Lauter, D.J. and Munns, D.N. 1986. Salt resistance of chickpea genotypes in solutions salinized with NaCl or Na_2SO_4. *Plant Soil* **95:** 271–279.

Lauter, D.J. and Munns, D.N. 1987. Salt sensitivity of chickpea during vegetative growth and at different humidities. *Aust. J. Plant Physiol.* **14:** 271–279.

Lauter, D.J., Munns, D.N., and Clarkin, K.L. 1981. Salt response of chickpeas influenced by N supply. *Agron. J.* **73:** 961–966.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

Pladys, D. and Rigaud, J. 1985. Senescence in french-bean nodules: occurence of different proteolytic activities. *Physiol. Plant.* **63:** 43–48.

Pladys, D., Barthe, P., and Rigaud, J. 1988. Changes in intracellular pH in french-bean nodules induced by senescence and nitrate treatment. *Plant Sci.* **56:** 99–106.

Poljakoff-Mayber, A. 1982. Biochemical and physiological responses of higher plants to salinity stress. In: *Biosaline Research; A Look to the Future.* A. San Prieto, ed. Plenum Press, New York, pp. 311–318.

Rai, R. and Prasad, V. 1983. Salinity tolerance of *Rhizobium* mutants: growth and relative efficiency of symbiotic nitrogen fixation. *Soil Biol. Biochem.* **15:** 217–219.

Salem, S.H., Gewaily, E.M., and EL-Zamik, F.I. 1981. Effect of salinity on the symbiosis of root nodule bacteria and braod bean plant. *Agrokemia es Talajtan* **30:** 83–89.

Sheoran, I.S., Kaur, A., and Singh, R. 1988. Nitrogen fixation and carbon metabolism in nodules of pigeonpea (*Cajanus Cajan* L.) under drought stress. *J. Plant Physiol.* **132:** 480–483.

Singleton, P.W. and Bohlool, B.B. 1984. Effect of salinity on the nodule formation by soybean. *Plant Physiol.* **74:** 72–76.

Tu, J.C. 1981. Effect of salinity on *Rhizobium*-root hair interaction, nodulation and growth of soybean. *Can. J. Plant Sc.* **61:** 231–239.

Vance, C.P., Reibach, P.H., and Ellis, W.R. 1986. Proteolytic enzymes of legume nodules and their possible role during nodule senescence. In: *Plant Proteolytic Enzymes*, Vol. 2. M.J. Dalling, ed. CRC Press, Bocca Raton, FL, pp. 103–124.

Vassey, T.L. and Sharkey, T.D. 1989. Mild water stress of *Phaseolus vulgaris* plants leads to reduced starch synthesis and extractable sucrose phosphate synthase activity. *Plant Physiol.* **89:** 1066–1070.

Yousef, A.N. and Sprent, J.I. 1983. Effect of NaCl on growth, nitrogen incorporation and chemical composition of inoculated and NH_4NO_3 fertilized *Vicia faba* (L.) plants. *J. Expt. Bot.* **34:** 941–950.

Zahran, H.H. and Sprent, J.I. 1986. Effects of sodium chloride and polyethylene glycol on root-hair and nodulation of *Vicia faba* (L.) plants by *Rhizobium leguminosarum. Planta* **167:** 303–309.