Effect of NaCl Salinity on the Growth and the Nitrogen Status of Nodulated Cowpea (*Vigna sinensis* L.) and Mung Bean (*Phaseolus aureus* L.)

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Summary

Plants of *V. sinensis* and *P. aureus* were grown in the presence of five levels of NaCl ranging from 10 to 100 mM. *V. sinensis* grew better than control plants in the presence of NaCl even up to 75 mM, while *P. aureus* showed a decline in its growth even at 10 mM NaCl concentration. Nodulation of *V. sinensis* by *Rhizobium* cowpea strain no. 3200 occurred up to a salt concentration of 100 mM NaCl. When *P. aureus* was infected with *Rhizobium japonicum* strain no. G49, nodulation did not occur in the plants growing with more than 25 mM NaCl. Successful nodulation of both species in the presence of NaCl was associated with some kind of salt tolerance either of the host plant or of the *Rhizobium* partner. Cowpea *rhizobium* strain no. 3200 was found to tolerate the NaCl salinity better than *R. japonicum* strain no. G49. Total nitrogenase activity estimated by the amount of N fixed during the growing period was found to be positively correlated with $NaCl_{ext}$ application up to 75 mM in *V. sinensis*. However, this result was difficult to correlate with the ARA of the nodules measured at harvest. In contrast, salt application had a negative effect on N fixation by *P. aureus*.

Key words: Vigna unguiculata syn. sinensis, Phaseolus aureus, Rhizobium, salinity stress, nodulation, nitrogen status, acetylene reduction activity.

Introduction

The level and nature of salts have been found to affect leguminous plants differently in their growth, nodulation, nitrogen fixing capacity or in the accumulation of various organic and inorganic solutes (Imbamba, 1973; Balasubramanian and Sinha, 1976; Winter and Läuchli, 1982; Nukaya et al., 1982 a, 1982 b; Imamul Huq and Larher, 1983 a, 1983 b). Nodulated plants are likely to be more susceptible to salt toxicity than the non-nodulated kinds (Wilson, 1970). Varying salt sensitivity in different species of *Trifolium* has been reported by West and Taylor (1981) and Winter and Läuchli (1982). Balasubramanian and Sinha (1976) observed that mung bean was more susceptible to salinity than cowpea when the plants were stressed after nodulation. The sensitivity to salinity of *Rhizobium* and their respective hosts also vary. Subba

Abbreviations: ARA = acetylene reduction activity, N_T = Total nitrogen, N_S = Soluble nitrogen, $NaCl_{ext}$ = NaCl solution applied to the growth medium.

Rao et al. (1972) observed that while *R. meliloti* could tolerate salt concentration up to 3%, its specific host, *Medicago sativa*, was not nodulated even at 0.7% of NaCl. Almost similar observations were made recently by Sauvage et al. (1983). Salt sensitivity of bacteria is a determining factor in the nodulation process as nodule initiation depend on an effective root hair infection by the bacteria. Moreover, an effective transfer of photosynthates to the infecting bacteria is one of the prerequisites for nodule development. It is thus possible that nodulation in legumes suffering severe salt stress will suffer a set-back even if the plants can survive the salinity shock, because, under these circumstances, the plants utilise a significant portion of the photosynthates in the production of organic solutes to maintain their proper osmolarity (Imamul Huq and Larher, 1983 b).

The effect of NaCl salinity on the germination and growth and subsequently on the nodulation of the leguminous plants *V. sinensis* and *P. aureus* are studied in the present work. The plants were subjected to salinity stress ranging from low to very high NaCl concentrations from the onset of seed imbibation. A comparison of the susceptibility of the two plant species to NaCl salinity was made.

Materials and Methods

Plant material

Local varieties of cowpea [*Vigna unguiculata* (L.) Walp. Syn. *V. sinensis* (L.) Savi ex Hassk.] and mung bean (*P. aureus* Roxb.) seeds were purchased from a seed dealer in Dacca (Bangladesh). The cowpea seed used was the «Early season» variety and the mung bean was the «Lowland» variety.

The seeds were germinated directly on vermiculite in perforated (at the bottom) rectangular plastic pots of about 7 l capacity after having the grains surface sterilized with a 0.5 % KOCl for 5 min. The vermiculite was soaked in a nutrient solution containing 0.8 mM P, 2.4 mM K, 2 mM Ca, 0.8 mM Mg, 0.8 mM S, 4 mM Cl^-, 4 μM Fe, 32 μM B, 11 μM Zn, 0.94 μM Mo, 0.5 μM Cu, 0.34 μM Co and 11 μM Mn. For the salt treatments, NaCl at concentrations of 0, 10, 25, 50, 75 or 100 mM was mixed with the nutrient solution and added to the vermiculite. The treatments are designated here as control, S_1, S_2, S_3, S_4 and S_5 respectively. The salinity stress was applied from the beginning of germination and continued until the end of the experiment.

Rhizobium cowpea, strain no. 3200 and *Rhizobium japonicum*, strain no. G49 were obtained from the Soil Microbiology Laboratory of INRA, Dijon, France (courtesy, Miss. N. Amarger). The bacteria were brought into suspension in distilled water and added to the rhizosphere of each plant. *V. sinensis* was infected with the strain no. 3200 and *P. aureus* with the strain no. G49. *V. sinensis* was harvested 40 days after germination and *P. aureus* after 45 days. On each occasion, the bacteria were added to the rhizosphere between 1 and 2 weeks after the seedling emergence.

The plants were grown in a controlled environment growth chamber at a daylight intensity of 30 W m^{-2} at leaf level, and day and night temperatures of 26 ± 1 °C and 22 ± 1 °C respectively. The corresponding relative humidities were 70 and 80% under 14 h day and 10 h night.

Distilled water was added daily to maintain a constant moisture content in the rooting medium. Both nutrient solutions and NaCl were renewed weekly. At harvest, nodules and roots were rinsed three times (1 min each) in distilled water. Fresh weights of above ground shoots and roots as well as those of the nodules were noted. Dry matter was determined after drying at 85 °C for 48 hours.

Bacterial culture

Bacteria were cultured in test tubes on a reciprocal shaker at 30 °C in the presence of the same concentration of NaCl as used for the plants. The basal medium was that of Wright (1925) modified by Pochon (1954). 4 replicates each of the treatments were made. The bacterial growth was noted up to an incubation period of 152 h. The growth is indicated as the changes in optical density at 580 nm over zero time. Values presented in the text are the averages of 4 individual replicates.

Analyses

Oven dried samples were ground in a ball mill and passed through a 0.5 mm sieve. These powdered samples and fresh nodules were extracted in the cold with 80 % ethanol. The crude extracts were evaporated in a vacuum and the residue dissolved in distilled water. This aqueous extract was used to determine the soluble –N contents. Total N was determined colorimetrically in the micro-Kjeldahl digest of the dry samples of plants and nodules following the method of Mann (1963). Soluble N was determined in the micro-Kjeldahl digest of the aqueous extract.

Acetylene reduction activity of the root nodule system was determined by gas chromatography and the results are expressed as the amounts of C_2H_4 produced per g dry matter of nodules per h.

The amount of N fixed by the plants was calculated by deduction of the N content of 100 plants at harvest to those of 100 grains.

Results

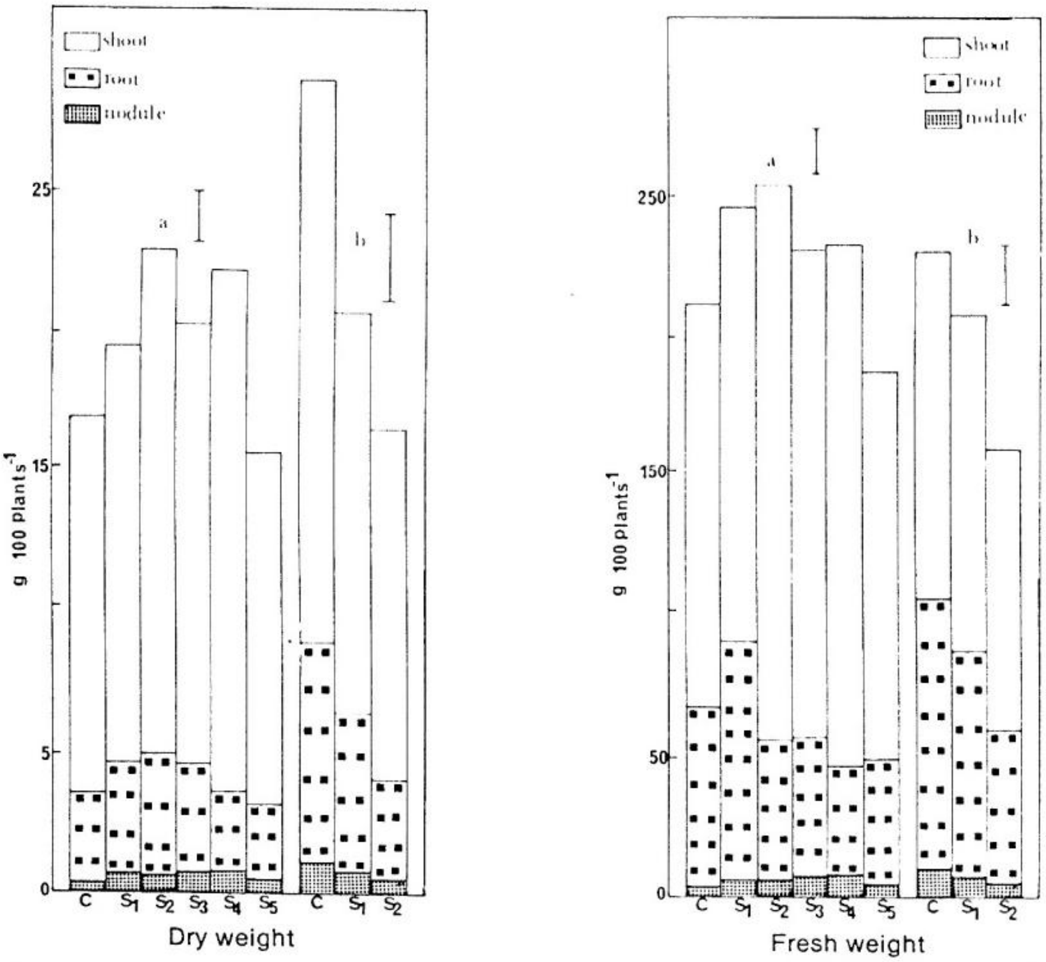
Effect of NaCl on the germination, growth and nodulation of V. sinensis and P. aureus

Germination: Seeds of both species tolerated well the range of salinity used in the present study. The seeds of *P. aureus* germinated earlier than those of *V. sinensis* in all the salt treatments. Though the germination and emergence of seedlings occurred almost at the same time in all the treatments, their growth rate was slower in the treatments S_3, S_4 and S_5. The salt treated plants, especially those treated with 50 mM and above, were shorter. This was more pronounced for the plants of *P. aureus.*

Plant growth: The plants of *V. sinensis* grew better than *P. aureus* in presence of $NaCl_{ext}$. Both fresh and dry weights of shoots of the former species were higher than those of the control plants up to a salt treatment of 75 mM (Fig. 1 a). The decrease in fresh weight at S_5 was very negligible (less than 5 %) while that for dry weight was about 7 % of the control. The root fresh weights were variable. The shoot/root ratio for fresh weights was 2.5 for the control and 3.0 for S_5 plants and those for dry weights were 4.0 and 4.6, respectively, for the two treatments.

Plants of *P. aureus* showed a decrease in both fresh and dry weights in their shoots and roots in the presence of NaCl (Fig. 1 b).

Nodulation: The plants of *V. sinensis* in all the NaCl treatments were well nodulated. Salt concentration seemed to have very little adverse effect on the number, sizes and weights of the nodules, except at S_5 where the number of nodules were relatively lower than in other $NaCl_{ext}$ treatments. Both fresh and dry weights of the nodules of salt-treated plants were higher than those of the control plants (Fig. 1 a). How-



a b

Fig. 1: Dry and fresh weights of different organs of nodulated plants of *V. sinensis* and *P. aureus* The small bars represent L.S.Ds at 5 % level of significance.

ever, in S_4 and S_5 plants the proportion of nodulated plants was less while the proportion of inactive nodules was greater.

Nodulation in *P. aureus* occurred only in the plants suffering salinity stress up to 25 mM $NaCl_{ext}$. However, the weights of the nodules per 100 plants decreased with salinity (Fig. 1 b). All the parameters concerning the nodule growth (size, number and weights) were better in the control plants. It should be mentioned that the nodules of *P. aureus* were much larger (5–7 mm) than those of *V. sinensis* (1–2 mm).

Acetylene Reduction Activity (ARA) of the detached root-nodule system collected from the salt treated plants of V. sinensis and P. aureus

The C_2H_4 produced were measured one hour after exposing the system to C_2H_2 (Table 1).

Table 1: Acetylene Reduction Activity (ARA) of the detached root-nodule systems of the salt treated plants of *V. sinensis* and *P. aureus*. Results are expressed as μmol $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ d.m. of nodules.

Symbiotic partners	mM NaCl in the external medium					
	0	10	25	50	75	100
V. sinensis × *R. cowpea* strain no. 3200	3.1	3.1	3.2	3.6	11.2	20.8
R. aureus × *R. japonicum* strain no. G49	412.9	551.7	292.5	–	–	–

The nodules of *V. sinensis* treated with 75 and 100 mM $NaCl_{ext}$ were more active than the others. The ARA at 100 mM $NaCl_{ext}$ was 7-fold greater than the control. For the nodules of *P. aureus*, values were found to be several-fold greater.

Nitrogen status (total N and soluble N) of the plants of V. sinensis and P. aureus at harvest

The results for N_T and N_S, expressed as mg 100 plants^{-1} are presented in Table 2. With increasing concentration of $NaCl_{ext}$, the N_T in shoots of *V. sinensis* increased up to S_3, while in the roots this tendency was maintained up to S_4. On the other hand, the soluble N content increased up to S_3 for both the shoots and roots. The values for N_S in the nodules of this plant showed a decreasing tendency with increasing salt treatment, the lowest content of N_S being observed at S_4. At the last two levels of $NaCl_{ext}$ treatments, the ARA were relatively higher.

For *P. aureus*, the N status in all parts of the salt-treated plants was lower compared to the control plants (Table 2). The same was true for the nodules. The nodules at S_1 had the lowest quantities of both N_T and N_S although they were found to have a high ARA.

Table 2: Amounts of total N (N_T) and soluble N (N_S) (mg 100 plants^{-1}) in salt stressed plants of *V. sinensis* and *P. aureus*.

Symbiotic partners	Plant Organs	mM NaCl in the external medium											
		0		10		25		50		75		100	
		N_T	N_S	N_T	N_S	N_T	N_S	N_T	N_S	N_T	N_S	N_T	N_S
V. sinensis ×	Shoot	392.3	165.4	440.0	167.2	448.7	176.3	455.2	166.9	375.3	160.2	175.7	131.0
R. cowpea	Root	48.9	19.5	58.0	23.2	64.8	21.5	66.7	21.6	50.0	17.2	40.0	13.6
	Nodule	N.D.	32.5	N.D.	15.9	N.D.	17.4	N.D.	25.2	N.D.	12.5	N.D.	13.3
P. aureus ×	Shoot	383.2	109.5	237.6	76.4	174.6	33.4						
R. japonicum	Root	79.6	44.8	55.3	25.6	30.7	7.5	–		–		–	
	Nodule	33.7	11.9	15.1	4.9	16.7	5.0						

N.D.: not determined.

Growth of R. japonicum and Cowpea rhizobium in the presence of NaCl

The two *rhizobia* were grown *in vitro* in the presence of the same NaCl concentrations as used for the plants. Neither of the strains tolerated more than 50 mM NaCl in their growth media (Table 3). Bacterial growth stopped after 6 h of incubation in both cases at 75 and 100 mM NaCl treatments. At the end of 152 h of incubation both the strains were still in their exponential phase. In fact, the strain of cowpea rhizobium which had a longer lag phase than *R. japonicum*, grew almost equally well in 25 mM NaCl as in the control. At 152 h of incubation, growth reduction in cowpea rhizobium was only 10 % of control while that for *R. japonicum* was more than 50 %. This may indicate some kind of salt tolerance of the former strain of *Rhizobium.*

Table 3: Growth of pure bacterial cultures of *Rhizobium* cowpea, strain no. 3200 and *Rhizobium japonicum*, strain no. G49 at 152 h of incubation in presence of various concentrations of NaCl. The values are the difference in O.D. from zero time measured at 580 nm. (a) O.D.; (b) % inhibition from control.

	R. cowpea		*R. japonicum*	
mM NaCl	a	b	a	b
0	0.38	0	0.32	0
10	0.37	2.6	0.26	19.7
25	0.34	10.5	0.16	50.1
50	0.07	81.6	0.12	63.1
75	0.02	94.7	0.03	90.7
100	0.02	94.7	0.02	93.8

Discussion

The results obtained in the present study clearly show that nodulated leguminous plants have quite different adaptive potentials to the adverse conditions of excess NaCl in their growth media. It is apparent that *V. sinensis* tolerates NaCl salinity much better than *P. aureus.*

Increases in all the growth parameters, especially that of dry matter production up to 50 mM $NaCl_{ext}$ treatment in the plants of *V. sinensis* was not solely due to a passive accumulation of Na^+ and Cl^- since the total N status of the plants was also improved in the presence of NaCl. This might be of interest for a plant accumualting storage proteins in its seeds.

Such a favourable situation is possible only if NaCl has a positive effect on N_2 fixation during the whole growing period. Thus, the amount of N_2 fixed increased from 240 mg in the control plants to 321 mg N 100 $plants^{-1}$ in the 50 mM NaCl treated plants. Salt tolerance of *V. sinensis* is also evident from the fact that at 75 mM $NaCl_{ext}$ treatment the amount of N_2 fixed was only slightly decreased (224 mg N 100 $plants^{-1}$), while at 100 mM $NaCl_{ext}$ the fixation was drastically reduced to only 15 mg N 100 $plants^{-1}$. These values give a complete picture of the nitrogenase activity of the nodules induced by the cowpea rhizobia on the roots of this species. In fact, the nitrogenase activity determined by an ARA assay on the detached root-nodule systems was not negatively affected by NaCl in the growth media. It can be assumed that at the time of measurement the ARA was relatively higher in the plants fixing less nitrogen during the whole growing period. Naturally the question arises of whether this determination might be an artefact of the method used or might be associated with a quite different physiological state of the nodules. Clearly, this needs further study.

In *P. aureus* the decrease in dry matter production with increasing salinity is closely associated with a decrease in the nitrogen status. This is directly related to the capability of this species for N_2 fixation under condition of salt stress. Thus, at 10 mM $NaCl_{ext}$ only 44 mg N_2 were fixed during the whole growing period while in the control plants the N_2 fixed was 244 mg. Here also, the values of fixed N_2 were rather inversely related to the ARA measurements. Moreover, the plants at 25 mM $NaCl_{ext}$, though nodulated, did not fix N_2.

Our results concerning the growth stimulation of *V. sinensis* by NaCl are in agreement with those of Balasubramanian and Sinha (1976), though in the latter case the $NaCl_{ext}$ was not same as in the present study. Differences in NaCl sensitivity among *Trifolia* species have been reported by Winter and Läuchli (1982) and West and Taylor (1981). However, in all these cases, the salinity stress was introduced to the plants already nodulated, which makes a comparison with our results difficult.

The question arises to know how could the plants benefit from the presence of NaCl in their growth media. Obviously, an effective nodulation of the salt-treated plants of *V. sinensis* by rhizobia cowpea was a prerequisite for avoiding N deficiency (a situation that occurred in the salt treated *P. aureus*). The very different results obtained with the two species under study suggest that nodulation in the presence of high $NaCl_{ext}$ depends largely on the extent of adaptability of the host plant to this adverse condition. It also depends on the *per se* salt resistance of the bacteria since they have to grow in the external media as well as in the root cytosol, both the media containing unusual concentrations of Na^+ and Cl^-. Thus, the cowpea rhizobium, which was found to tolerate high concentrations NaCl better than *R. japonicum*, was able to infect its host and produce effective nodules at higher $NaCl_{ext}$. In fact, although neither of the rhizobia strains could tolerate more than 50 mM NaCl in pure culture, *V. sinensis* was found to be nodulated even at 100 mM $NaCl_{ext}$, indicating that the bacteria benefit the adaptative potential of its host to NaCl. This observation is not in agreement with those reported by various authors (Subba Rao et al., 1972; Yadav and Vyas, 1973; Sauvage et al., 1983; Sauvage, personal communication) who have shown that effective nodulation under salt stress usually occurs at lower NaCl concentration than that which inhibits rhizobia growth in pure culture.

On the basis of our results with *V. sinensis*, which exhibits some kind of salt requirement for its growth, and those concerning the salt sensitivity of *P. aureus*, it can be suggested that the capability of the host plant to maintain a proper nutritional environment for the bacteria, even in the presence of high salinity is of major importance in nodulation and subsequent N_2 fixation. Obviously, this needs an adequate supply of photosynthates (Bergersen, 1971) and a sufficient rate of oxidation for these substrates via an efficient glycolytic pathway. Such a metabolic situation might arise due to an increased activity of phosphofructokinase and pyruvate kinase, two enzymes known to be activated by monovalent cations including Na^+ (Turner and Turner, 1980). In addition, as suggested by our results, N_2 fixation might require in a few cases a small quantity of Na^+. Increased ARA up to a 1% NaCl concentration has also been reported in the nodules of *Prosopis velutina* infected by *Rhizobium mesquite* (Hua, 1981). However, further studies are needed before a rational explanation can be offered for the reasons of efficient N_2 fixation by *V. sinensis* under a salt treatment of as much as 75 mM $NaCl_{ext}$.

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