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Toxic and Osmotic Effects of Salinity on Growth and Nodulation of *Medicago sativa*

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Abstract

Toxic and osmotic effects of NaCl on growth and nodulation of alfalfa plants (*Medicago sativa* L. cv. Gilboa) were studied in a controlled environment in a greenhouse. PEG (polyethylene glycol), NaCl, KCl, Na_2SO_4, K_2SO_4 or $CaCl_2$, all at 130 mOsm, had reduced plant dry matter production by 50% after 55 days of growth. Nodule number was mainly affected by the toxicity of the salts, rather than by the osmotic stress induced by the PEG treatments. Delaying NaCl application for 33 days resulted in increased nodule number and nodule weight as compared with early salt application. Nitrogenase specific activity and nodule development (as defined by the average weight of one nodule), were not significantly affected by any of the above iso-osmotic treatments applied either early or late, but nodule weight was affected by PEG. The reduction in nodule number, induced by the above salts at 250 mOsm in the root zone, was 70, 65, 55, 22 and 2% for Na_2SO_4, NaCl, KCl, K_2SO_4 and $CaCl_2$, respectively. Light microscopy study of roots of 4-day-old seedlings grown on Fåhraeus slides showed that, at 200 mOsm NaCl, there was a significant reduction in root-hair length and in the proportion of root hairs containing an infection thread. This effect was not observed with PEG. The results indicate that under salt levels up to 200 mOsm (90 mM NaCl), initiation of alfalfa nodules is more sensitive to the toxic effects of sodium and chloride ions than to the osmotic stress induced by the salt.

Keywords: Alfalfa, growth, *Medicago sativa*, nitrogen fixation, nodulation, osmotic effects, *Rhizobium*, salt

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1. Introduction

Plants under salt stress, are subjected to two related but different influences: (1) osmotic stress, due to the low water potential of the medium, and (2) toxic stress, generated by the injury caused by the toxic ions that accumulate in the plant tissue. Both effects seem to affect crop growth and yield (Redmann, 1974). But, it is often difficult to separate and assess the relative importance of ion excess and water deficit, or to determine in which tissue the primary effect is located (Greenway and Munns, 1980).

Legumes depending on symbiotic N_2 fixation seem less tolerant to salt than when they are grown on mineral nitrogen (Bernstein and Ogata, 1966; Wilson 1970). Applications of salt and osmotic stress reduced total plant N, but the specific activity of nitrogenase was not restricted (Wilson, 1985). Singleton and Bohlool (1984) reported that nodule initiation in soybean was adversely affected by NaCl concentrations that are inhibitory neither to rhizobial survival nor to the colonization of the root surface. Similarly, drought reduces nodule formation in *Trifolium subterraneum* without affecting rhizobial numbers in the soil (Worrall and Roughley, 1976). In *Vicia faba*, both osmotic (polyethylene glycol, PEG) and toxic (NaCl) stresses interfered with root-hair development and caused considerable reduction in the infection process (Zahran and Sprent, 1986). The general conclusion from the various studies is that root nodule formation in the legume-*Rhizobium* symbiosis is more sensitive to each type of stress than growth and survival of either symbiont alone in the presence of mineral nitrogen (Bernstein and Ogata, 1966; Subba-Rao et al., 1972). Although both stresses affect plant development to the same extent, they may also adversely affect the physiology of nodulation and N_2 fixation, and this possibility has not yet been studied in detail.

Relative to other forage legumes, alfalfa is moderately sensitive to salt stress (Downton, 1984), and nodulation is more resistant to salinity than that of soybean at similar NaCl levels (Bernstein and Ogata, 1966). Despite the fact that NaCl application to alfalfa roots leads to a reduction in root-hair expansion and nodule development (Lakshmi-Kumari et al., 1974), to the best of our knowledge no attempts have been made to see whether these alterations relate to the osmotic or to the toxic effects of NaCl application. The purpose of the present study was to investigate the differential capacity of the toxic and osmotic stresses to affect growth and nodule formation of alfalfa plants under saline conditions. In the present paper we show that even though NaCl and PEG applications reduced plant growth to the same extent, nodule initiation was reduced by the toxicity of the salt rather than by the osmotic stress induced by the PEG.

2. Materials and Methods

Growth conditions

Seeds of alfalfa (*Medicago sativa* L. cv. "Gilboa") were surface-sterilized with 70% ethanol for 7 min, rinsed five times in sterile distilled water, and allowed to germinate on 1% water agar for 20 h. Ten germinated seeds were planted in a sterile-modified Leonard jar (Vincent, 1970) containing sand, and inoculated with *R. meliloti* strain 102F28 (10^8 colony forming units (c.f.u.) per seedling). Plants were grown under semi-controlled conditions in a greenhouse at 27/21°C (day/night) with natural daylight supplemented by fluorescent [Sylvania, F40W/CW/T-12 (white light) + Growlight, Philips, 40W (blue light)] to provide a 14 h photoperiod. Plants were thinned out to five plants per jar, 14 days after planting. Salts or PEG (MW 8000, Sigma) were applied 72 h after planting (early application) by adding each of them to the N-free nutrient solution (KH_2PO_4, 0.5 mM; K_2SO_4, 1.0 mM; $MgSO_4$, 1.0 mM; $CaSO_4$, 1.0 mM and 1 ml per liter of micronutrients adapted from Kapulnik et al. (1989). Salts or PEG were added to bring the osmotic levels to 0, 70, 130, 200 or 250 mOsm (Table 1). Preliminary experiments conducted prior to the experiments reported in this work revealed that washing and reloading the soil in the pots with water and PEG solution, respectively, affected plant growth significantly. After verifying that the PEG at this MW was not taken up by the plants, the concentration of PEG solution to be added to the soil was determined based on the water capacity of the medium and its dry weight. The late application was carried out 33 days after planting, with the same concentrations of salts; there was no late application of PEG. Moisture level in the soil was maintained to a constant weight during the experiment by using sterile distilled every following day.

Table 1. Relationship between concentration and osmolarity for various salts

	mOsm			
	70	130	200	250
Salt, concentration in mM				
NaCl	30	60	90	120
KCl	25	50	75	100
K_2SO_4	15	30	45	60
Na_2SO_4	19	38	56	75
$CaCl_2$	25	50	75	100
PEG, concentration in %	8	12	14	17

Experiments were carried out in a completely randomized block design with six replicates, each containing two Leonard jars with 5 plants. Salinity concentrations were monitored in the rooting media by means of weekly measurements of electrical conductivity in the drainage solution taken from two replicates of each treatment. Salts were added as required to maintain the specific osmolality in the jars. Once weekly, all treatments were thoroughly washed with sterile distilled water, to prevent the build-up of high concentrations of salts in the jars. Experimental plants were allowed to grow until those in the non-saline treatment reached a blooming shoot rate of 20%; the roots of all plants in the experiment were then harvested and analyzed in a separate group for each replicate.

Observations by light microscopy

Slides were prepared as described by Fåhraeus (1957) and placed in test tubes (40 cm tall by 4 cm in diameter) containing 20 ml of the N-free nutrient solution. Pregerminated seedlings (see above) were inserted under the cover slip and transferred to a growth chamber with 14 h day: 10 h night and 27:20°C for 16 h. After this short incubation, salt or PEG treatments were applied. Twelve hours later, the assemblies were inoculated with 10^6 c.f.u. of *R. meliloti* cells per plant, and the tubes were again placed in the growth chamber. After 72 h the numbers of root hairs and infection threads were counted under a light microscope after staining by methylene blue (Vasse and Thruchet, 1984) of root sections developed after inoculation.

Measurement of Rhizobium growth

The effects of different salt concentrations on the growth of Rhizobium meliloti, strain 102F28 were determined in Yeast Extract-Mannitol broth (Vincent, 1970). Cultures were incubated in a shaking waterbath at 29°C and absorbance at 420 nm was measured by means of a spectrophotometer.

Plant analysis

At harvest, shoots were cut off at the cotyledonous node, and the nitrogen fixation activity of the detached root systems was determined by acetylene reduction assay (Hardy et al., 1968) using gas chromatography (Varian, model 3700 - flame ionization). Results were expressed as nmole ethylene produced per mg nodule dry weight and hour. Nodules were detached from the roots, counted, and oven-dried at 70°C for 72 h.

All plant samples were dried under the same conditions and ground to pass through a 1-mm screen. After wet digestion with concentrated H_2SO_4 at 180°C, nitrogen was determined by the micro-Kjeldahl method (Vincent, 1970); Na^+ and K^+ by flame photometry (Corning, model 410); Cl^- by chloridometer (Haake-Büchler, automatic titration); and Ca^{2+} by atomic emission spectroscopy (Spectroflame, Spectro). All data were tested for treatment effects by analysis of variance (Little and Hills, 1978).

3. Results

Plant dry weight accumulation at harvest (55 days after planting) was significantly influenced by early NaCl application at concentrations above the equivalent of 130 mOsm (Fig. 1). A similar reduction in plant growth was obtained after PEG application at the same osmotic levels. By contrast, if the application of NaCl was delayed until 33 days after planting, plant growth at harvest was not significantly affected (Fig. 1).

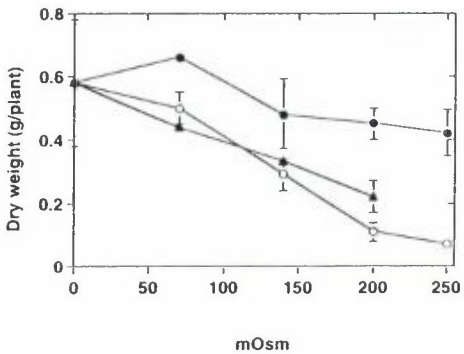

Figure 1. Total dry weight of 55-day-old *M. sativa* cv. Gilboa plants grown at different osmotic levels induced by NaCl (empty circle) and PEG (filled triangle) at early applications or NaCl (filled circle) at a late application. Plants were grown under N_2-dependent conditions, and early and late applications were carried out 3 and 33 days after planting, respectively. Vertical bars indicate +/- standard error. Each value represents the mean of six replicates, each of which contained five plants.

According to measurements of the doubling time (4.3 and 4.1 h under saline and non-saline conditions, respectively), growth and survival of *R. meliloti* strain 102F28 in culture, was not affected by the application of 340 mOsm of NaCl. A significant decrease in *Rhizobium* cell growth was obtained in 680 mOsm NaCl, which increased the doubling time to 7 h.

Early application of NaCl resulted in a significant reduction in nodule number but not in nodule mass (Figs. 2a, b). At comparable osmotic levels, PEG application did not reduce nodule number although it decreased nodule mass. It is interesting to note that the number of nodules at a PEG level equivalent to 70 mOsm was significantly greater than in the non-stressed controls (Fig. 2a). The total nodule mass of PEG-treated plants was generally lower than that of NaCl-treated plants. In spite of the notable distinct effects of early applications of PEG and NaCl on nodule number and mass, no significant effects on N concentration in plants were found (Table 2). Application of NaCl treatment 33 days after planting (late application) had no significant effect on any of the above parameters at harvest.

Specific activity of nitrogenase (as μmole C_2H_4 (mg nodule dry weight)$^{-1}$ (h^{-1})) was significantly affected by PEG, but not by similar osmotic levels induced by NaCl (Fig. 3). PEG levels of 70, 130 and 200 mOsm increased nitrogenase specific activity significantly by 35, 59 and 35%, respectively. NaCl concentrations equivalent to 130 and 200 mOsm at the early and late salt applications did not significantly affect the nitrogenase specific activity as compared with the non-saline treatment. The nitrogenase specific activity was

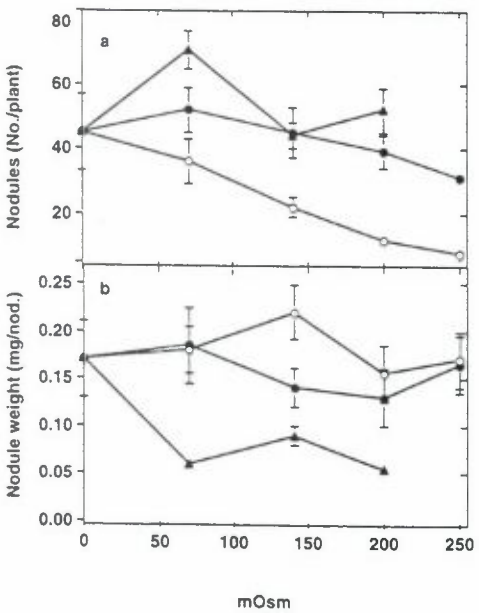

Figure 2. Nodule number (a) and mean nodule weight (b) of 55-day-old *M. sativa* cv. Gilboa plants grown at different osmotic levels induced by NaCl (empty circle) and PEG (filled triangle) at early applications or NaCl (filled circle) at a late application. Otherwise as for Fig. 1.

Table 2. Effect of early applications of NaCl and PEG on ion concentrations in 5 week-old alfalfa plants

	mOsm	Na^+	Cl^-	K^+	Ca^{2+}	N
		mg/g dry weight				
NaCl	0	2.0	4.3	29.6	16.9	25.2
	70	10.3	13.1	24.4	14.9	27.1
	130	17.0	20.0	19.0	13.9	24.0
	200	21.8	25.4	18.1	13.6	22.8
	250	36.2	–	13.0	12.0	24.0
L.S.D. (0.05)		7.7	4.8	3.2	1.9	4.7
PEG	0	2.5	5.5	25.3	–	31.0
	70	2.6	5.4	28.5	–	30.9
	130	2.6	6.0	27.5	–	31.2
	200	2.5	6.6	33.5	–	32.9
	250	–	–	–	–	–
L.S.D. (0.05)		0.6	0.9	6.6	–	2.3

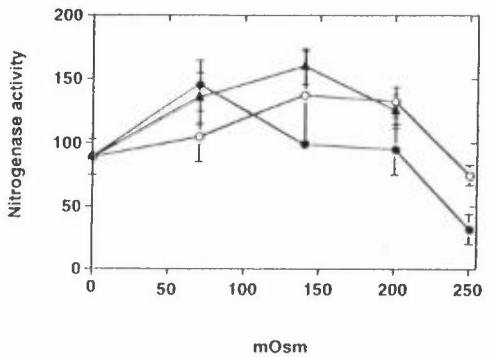

Figure 3. Specific activity of nitrogenase of 55-day-old *M. sativa* cv. Gilboa plants grown at different osmotic levels induced by NaCl (empty circle) and PEG (filled triangle) at early applications or by NaCl (filled circle) at a late application. Activity expressed as μmole C_2H_4 (mg nodule dry weight)$^{-1}$ h^{-1}, otherwise as for Fig. 1.

reduced by both early and late NaCl applications at 250 mOsm, but the reduction was significant only for the late NaCl treatment (Fig. 3).

The reduction in plant dry weight of salt-treated plants in the early

application treatment was associated with significantly higher concentrations of Na^+ and Cl^- and lower concentrations of K^+ and Ca^+ (Table 2). When the same osmotic levels were induced by PEG, comparable values of plant dry weight were obtained, but no significant differences in Na^+, Cl^- and K^+ concentrations were found (Table 2).

Equal osmotic levels of NaCl, KCl, Na_2SO_4, K_2SO_4 and $CaCl_2$ reduced plant growth significantly, and the reductions increased with increasing osmotic potential of the added salt (Fig. 4a). After 55 days of growth, 130 mOsm of each of the salts tested had reduced plant dry weight by 50%, as compared with non-stressed controls. Nodule number and nodule specific weight were differently affected by the different salts: Na_2SO_4, NaCl and KCl reduced the nodule number significantly by 55, 37 and 37%, respectively, whereas K_2SO_4 and $CaCl_2$ application resulted in non-significant reductions in nodule number by 25 and 23 %, respectively (Fig. 4b). In contrast, nodule specific weight was significantly reduced after K_2SO_4 and $CaCl_2$ application (from 0.16 mg/nodule in non-stressed controls to 0.06 and 0.05 mg/nodule in plants treated with 130 mOsm of K_2SO_4 or $CaCl_2$, respectively), but did not change much at any of the osmotic levels induced by application of Na_2SO_4, NaCl or KCl. At the highest osmotic level (250 mOsm), all salts except for $CaCl_2$ and K_2SO_4 reduced the nodule number significantly, by 71, 71 and 56% for Na_2SO_4, NaCl and KCl, respectively (Fig. 4b). Nitrogenase specific activity was not significantly affected by any level of the salts tested (data not presented).

Salt application altered root hair morphology and infection thread formation of 4-day-old alfalfa seedlings. A significant reduction in the length of root hairs was found in NaCl-treated plants as compared with non-treated controls (Fig. 5a). However, similar osmotic levels induced by PEG did not reduce root hair length (Fig. 5a). Salt-stressed root hairs were thicker and shorter than those of PEG-treated or control plants (compare Fig. 6b with Figs. 6c and 6a, respectively).

At a level of 200 mOsm induced by NaCl, the proportion of root hairs bearing infection threads was reduced by 66% and root hairs were shorter by 50%, both relative to non-treated control plants (Figs. 5a, 5b). However, application of PEG at a similar osmotic level did not affect the proportion of the infected root hairs or root hair length (Fig. 5b). At this osmotic level, neither NaCl nor PEG reduced root hair formation per mm of main root length significantly (data not shown).

4. Discussion

Growth and nodule formation of alfalfa are influenced differently by

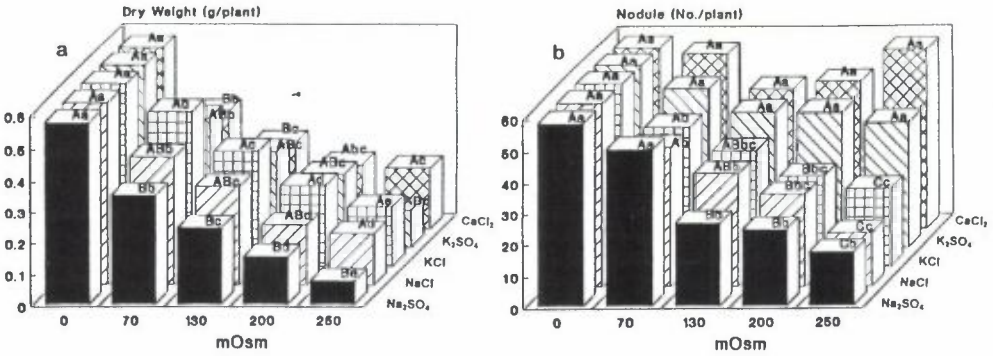

Figure 4. Effects of different osmotic levels of Na_2SO_4, NaCl, KCl, K_2SO_4 and $CaCl_2$ on alfalfa plant dry weight (a) and nodule number (b). Values for a single treatment followed by the same capital letter did not differ significantly ($P<0.05$) among osmotic levels within a salt treatment, whereas values followed by the same small letter did not differ significantly ($P<0.05$) among salts within the osmotic level treatment . Each value represents the mean of six replicates, each of which contained five plants.

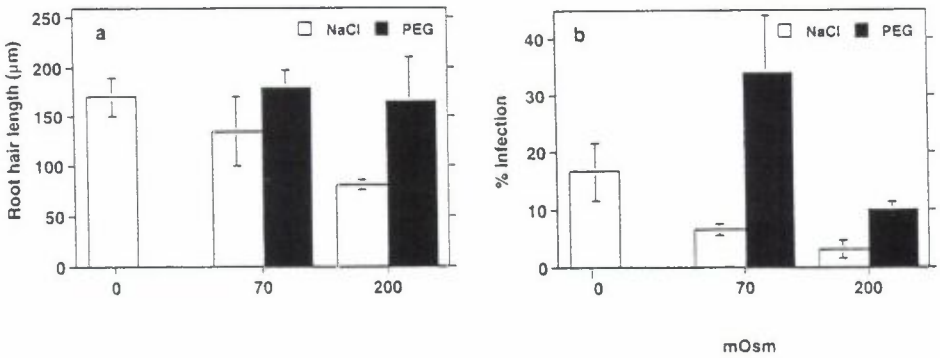

Figure 5. The effect of different osmotic levels of NaCl and PEG on root hair length (a) and the proportion of root hairs bearing infection threads (b) in 5-day-old alfalfa seedlings. Plants were grown on Fåhraeus slides and measurements were made 72 h after inoculation. Values are means of 16 plants in four replications vertical bars indicate +/– standard error.

applications of NaCl and PEG. A 50% reduction in both plant growth and nodule formation was obtained at NaCl levels equivalent to 130 mOsm (Fig. 1). At the same osmotic levels induced by PEG application, although a

comparable reduction in plant growth was obtained, nodule formation was not affected at all (Fig. 2a).

The fact that salt and PEG applications had comparable effects on alfalfa growth (Fig. 1) indicates that osmotic stress is the major suppressor of normal plant development. According to Nieman (1980), the energy required for salt resistance or adaptation is obtained mainly at the expense of growth. Moreover, osmotic stress affects photosynthate availability, which affects all stages of the *Rhizobium*-legume symbiotic associations (Sprent, 1976; Williams and De-Mallorca, 1984). However, in the present investigation, N concentration in the plants did not change significantly (Table 2). In fact, the efficiency of nitrogenase activity was not reduced during salt treatments (Fig. 3), as was indicated by the relatively small changes in N concentration compared with the non-stressed controls (Table 2). Similarly, Sanchez-Diaz et al. (1982) and Singleton and Bohlool (1984) found that nodule function was tolerant of low to moderate salinity levels and showed only slight impairment at NaCl concentrations up to 80 mM (170 mOsm). This suggests, that although salinity may reduce total carbohydrate availability in the plant, at the salt levels used in the present work, the energy required for N fixation activity was not the main limiting factor.

Although salinity reduced nodule number considerably (Fig. 2a), specific mean weight per nodule was not affected by NaCl applications up to 250 mOsm (Fig. 2b), indicating that once a nodule was initiated, its development was not impaired. In contrast, high osmolality induced by PEG, reduced specific mean weight per nodule significantly without affecting the nodule number (Figs. 2a, b). This difference in alfalfa plant response to the two kinds of stress implies that salt toxicity impaired nodule formation (initiation) specifically, whereas osmotic stress did not affect nodule initiation, but reduced its continued development, probably as a result of photosynthate limitations (Sprent, 1976).

Lakshmi-Kumari et al. (1974) reported that low NaCl levels reduced alfalfa root hair curling, deformation and shepherd's crook formation, all of which are characteristic of the early phases of nodule initiation. Similarly, Singleton and Bohlool (1984) concluded that in soybean, salt inhibits nodule initiation but not the ability of the rhizobia to colonize the root. These results support our observation that infection thread formation is highly susceptible to NaCl toxicity (Fig. 5) and can form a limiting step in nodule initiation. Whether this is the earliest event in *R. meliloti-M. sativa* symbiosis that inhibits alfalfa nodulation after salt application requires further investigation.

Application of a given concentration of a PEG solution to the growth media does not necessarily represent the actual osmotic level induced in the experimental system. Plant development and biomass obtained at the different

concentrations of PEG agreed well with the amended increments of PEG to the soil. PEG did not cause apparent morphological changes in the root hairs (Fig. 6c), and its role in nodulation processes remains unclear. In white clover, nodulation of plants was greatly stimulated after PEG application (Al-Mallah et al., 1990), but in other cases, increased PEG reduced numbers and function of nodules, e.g., in *Glycine max* (Williams and De-Mallorca, 1984) and in *Vicia faba* (Zahran and Sprent, 1986).

The inhibition of nodule formation associated with the presence of NaCl in the medium was apparently not related to the survival of *R. meliloti* in the soil. Strain 102F28 managed to grow at NaCl levels much higher than those that caused a 50% reduction in plant growth. This agrees with Subba-Rao et al. (1972) and Botsford (1984), who reported the survival of some strains of *R. meliloti* even at 3% NaCl. Similarly, Worrall and Roughley (1976) found the number of rhizobia in the rhizosphere to be unaffected after induction of drought stress.

Evidence that it is specifically the toxicity of NaCl to alfalfa roots that reduces the number of nodules, and that Na^+ may have a toxic ion effect, lies in the fact that iso-osmotic levels of salts, except those having Na^+ in their configuration, failed to reduce the nodule number significantly (Fig. 4b). A significant increase in plant Na^+ and Cl^- and decrease in Ca^{2+} and K^+ was found for NaCl-treated alfalfa (Table 2). Similar results have been obtained for *Medicago sativa* by Ashraf et al. (1986) and Kapulnik et al. (1989). These findings suggest several possible ways in which NaCl may affect the nodulation process. One mechanism could involve a direct response of the root-hair to the significant increase in Na^+ and Cl^- and decrease in Ca^{2+} and K^+ levels in the tissue (Table 2). Displacement of membrane-associated Ca^{2+} by Na^+ is a primary response of cotton root hairs to salinity and may occur primarily at the external surface of the plasmalemma (Cramer et al., 1985). The lack of Ca^{2+} in the culture medium induces abnormal root-hair development (Cormack, 1962). To date, it is unknown whether alfalfa root hairs respond significantly at the Na^+ level used in our study by such changes. However, in many legumes, root hairs are the target cells through which rhizobia enter the plant after specific interactions involving recognition, attachment and signalling processes, all of which may depend on host membrane properties and eventually affect nodule formation.

Several mechanisms may exist for controlling the amount of nodules induced on roots of alfalfa. Although one cannot determine from our experiments the predominant physiological and biochemical effects induced by salinity, clearly, the results reported here suggest that under salt levels up to 90 mM NaCl the nodulation process is more sensitive to the toxic than to the osmotic effects induced by salt.

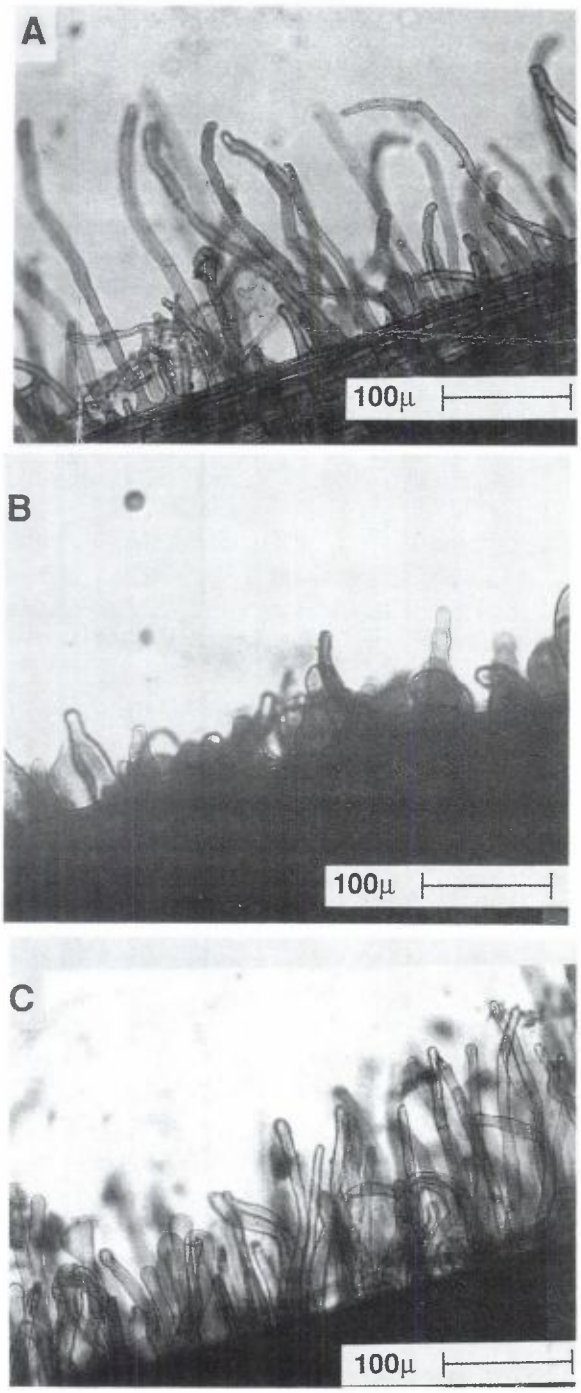

Figure 6. Light micrographs of *M. sativa* roots, showing the structure of root hair cells of non-treated control (A), NaCl (B) and PEG (C) treated plants 96 h after application of 200 mOSm solutions.

Abbreviations

mOsm = milliosmolality; PEG = polyethylene glycol; c.f.u. = colony forming units.

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