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Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*

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Abstract. The effects of sodium chloride and polyethylene glycol (PEG) on the interaction between *Rhizobium leguminosarum* strain 29d and root hairs of field bean (*Vicia faba* L. cv. Maris Bead) plants were investigated. Two levels each of NaCl (50 and 100 mol·m^{-3}) and PEG (100 and 200 mol·m^{-3}) were given at the time of root-hair formation. Scanning electron microscopy showed rhizobial attachment and colonization on root-hair tips. Adhesion of rhizobia in both lateral and polar orientation, sometimes associated with microfibrils, occurred mainly in crooks at the root-hair tips; most of the infections also occurred here. Bacterial colonization and root-hair curling were both reduced by stress treatments. Polyethylene glycol but not NaCl significantly reduced root-hair diameter. The proportion of root hairs containing infection threads was reduced by 30% under NaCl and by 52% under PEG. The structure of some of the root hairs, epidermal and hypodermal cells, as seen by light microscopy in ultrasections, was distorted as a result of NaCl and PEG treatments; cells showed plasmolysis and folded membranes. After three weeks of treatment, both NaCl and PEG inhibited nodule number by about 50% and nodule weight by more than 60%. It is concluded that the root-hair infection process in *Vicia faba* is impaired by NaCl and PEG treatments and this in turn results in fewer nodules being produced.

Key words: Nodulation – Root hair infection – Salt stress – *Vicia-Rhizobium* symbiosis.

Introduction

The legume-*Rhizobium* symbiosis is a highly integrated system. Soil-based stress may act on the symbiosis indirectly by reducing plant growth and available photosynthate, or by direct effects on the infection process and/or nodule function (Singleton 1983). In most crop legumes, infection by *Rhizobium* is an active process associated with root-hair curling, followed by penetration of the bacteria into the plant cell (Vance 1983). The vast majority of rhizobia which become attached to host root cells never produce an infection. In temperate legumes such as pea, lucerne or clover the proportion of infected hairs is usually between 5% and 10% and many of these infections are abortive (Dart 1977). Successful infections produce walled tubular structures (threads) carrying rhizobia intracellularly into the base of the infected hair and then through cell walls into adjacent cortical cells.

There are few reports on the effects of soil salinity on infection and nodule initiation. Lakshmi-Kumari et al. (1974) working with lucerne (*Medicago sativa*) found that 70 to 100 mol·m^{-3} NaCl led to a reduction in the number of root hairs; root-hair infections were reduced to a minimum by only 35 mol·m^{-3} NaCl. Further, they recorded that the few root hairs present appeared to be short, stubby and bulbous, and without the curling or shepherd's crook formation characteristic of the early phase of nodule formation. The failure of soybean to nodulate at 200 mol·m^{-3} NaCl was attributed by Tu (1981) to decreased rhizobial colonization. At higher salinity (255 and 340 mol·m^{-3} NaCl) using scanning electron microscopy, he demonstrated the shrinkage of soybean root hairs. Yousef and Sprent (1983) showed that NaCl affected nodulation, nodule development and activity, and concluded that there may also be effects on infection.

Reports of the effects of moisture deficits on the nodulation of legumes are scarce and have been mainly observational rather than physiological. A

Abbreviation: PEG = polyethylene glycol

reduction of soil moisture from 5.5 to 3.5% significantly decreased the number of infection threads and completely inhibited nodulation in *Trifolium subterraneum*, without affecting rhizobial numbers in the rhizosphere (Worrall and Roughley 1976). Osmotically induced leaf moisture stress (0.22 to 0.62 MPa) delayed nodulation and prevented the further development of nodule initials of soybean (Williams and Mallorca 1984), indicating an indirect effect of water stress on the nodule morphogenesis.

This study investigates effects of NaCl on root-hair growth, rhizobial infection and nodulation of *Vicia faba* and compares these effects with the matric and/or osmotic effects of polyethylene glycol (PEG).

Materials and methods

Growth of plants. Seeds of *Vicia faba* cv. Maris Bead (obtained from the National Seed Development Organization, Cambridge, UK) were surface sterilized in 5% sodium hypochlorite for 5 min, rinsed once with 70% ethanol and several times with sterilised distilled water, then germinated in sterilized Petri dishes under aseptic conditions at room temperature in the dark until radicles appeared (usually 3 d). Three healthy seedlings were transplanted into 13-cm-diameter pots containing 1.4 kg autoclaved coarse sand. As it has been suggested that infections develop most frequently in root hairs which emerge shortly after inoculation and that regions of the root where mature hairs are present at the time of inoculation are not susceptible to *Rhizobium* infection (Calvert et al. 1984), seedlings from some plants were removed daily and checked microscopically for root-hair formation. When hairs were seen to be forming, seedlings were inoculated with a young culture of *Rhizobium leguminosarum* strain 29d (from the Welsh Plant Breeding Station, Aberystwyth, UK) grown in Yeast Extract-Mannitol broth in a shaking waterbath at 27 °C. Three times every week pots were surface irrigated with nutrient solution containing: $CaSO_4.2H_2O$ (4 mol·m^{-3}), KH_2PO_4 (1.5 mol·m^{-3}), $MgSO_4.7H_2O$ (3.1 mol·m^{-3}), K_2SO_4 (0.75 mol·m^{-3}), $MnSO_4.4H_2O$ (1.0 mmol·m^{-3}), $CuSO_4.5H_2O$ (0.1 mmol·m^{-3}), $ZnSO_4.7H_2O$ (0.1 mmol·m^{-3}), H_3PO_4 (5 mmol·m^{-3}), NaCl (10 mmol·m^{-3}), $NaMoO_4.2H_2O$ (0.5 mmol·m^{-3}), $CoSO_4.6H_2O$ (0.02 mmol·m^{-3}) and $C_6H_5O_7Fe.5H_2O$ (5 mmol·m^{-3}).

Treatment with NaCl and PEG. At the time of root-hair formation and inoculation, NaCl or PEG (average MW 6000–7500; BDH Chemicals, Poole, Dorset, UK) was applied to the soil at the following concentrations: 50 and 100 mol·m^{-3} for NaCl and 100 and 200 mol·m^{-3} for PEG. As NaCl is known to be absorbed by *V. faba* (Yousef and Sprent 1983), 50 ml of each level were applied three times every week. On the other hand, since high-molecular-weight PEG is at most only slightly absorbed by plants (Lawlor 1970; Sánchez-Diáz et al. 1982), only one 50-ml application of the required level was given. The same volume of distilled water was applied to control pots and moisture content of the sand adjusted daily by weight and kept at water-holding capacity, about 10%. Plants were grown in March–April in a greenhouse maintained at 23±4 °C. The average natural daylenth of 11 h supplemented with light from fluorescent lamps to give a 14-h photoperiod. Replicate pots for each treatment combination (including harvest dates) were used and arranged in three randomized blocks. Plants were harvested daily for 8 d commencing 1 d after NaCl and PEG treatments.

Sampling of plants. Main roots were harvested from 1–6 d and lateral roots from 3–8 d after commencement of treatments. One-cm pieces of root were fixed in 4% glutaraldehyde in 100 mol·m^{-3} Na-phosphate buffer, pH 7, and kept at room temperature until the time of processing. After three weeks of treatment, nodules were harvested, counted and their fresh and dry weights determined.

Sections for light microscopy. Fixed samples were rinsed in buffer for 30 min, then passed through a graded ethanol:water series to pure ethanol, embedded in LR white resin (London Resin Co., Basingstoke, Hampshire, UK; Gillett and Longhurst 1982) and left overnight at 70 °C for polymerization. Blocks were sectioned (1–4 µm thickness) in a Sorval M2-2 ultramicrotome (Ivan Sorval Inc., Norwalk, USA). Sections were heat-fixed on clean slides and stained with 0.1% Toluidine Blue 0 in 1.0% (w/v) sodium borate.

Scanning electron microscopy. Samples were treated as above to the stage of pure ethanol, then passed through an ethanol-freon series, before drying in a Polaron series II critical-point drier (Polaron Equipment, Watford, Herts., UK). They were then mounted on aluminium stubs, coated with gold-palladium in a Polaron E 5100 coating unit and examined in a scanning electron microscope (JSM-35; Jeol, London, UK) at 25 kV.

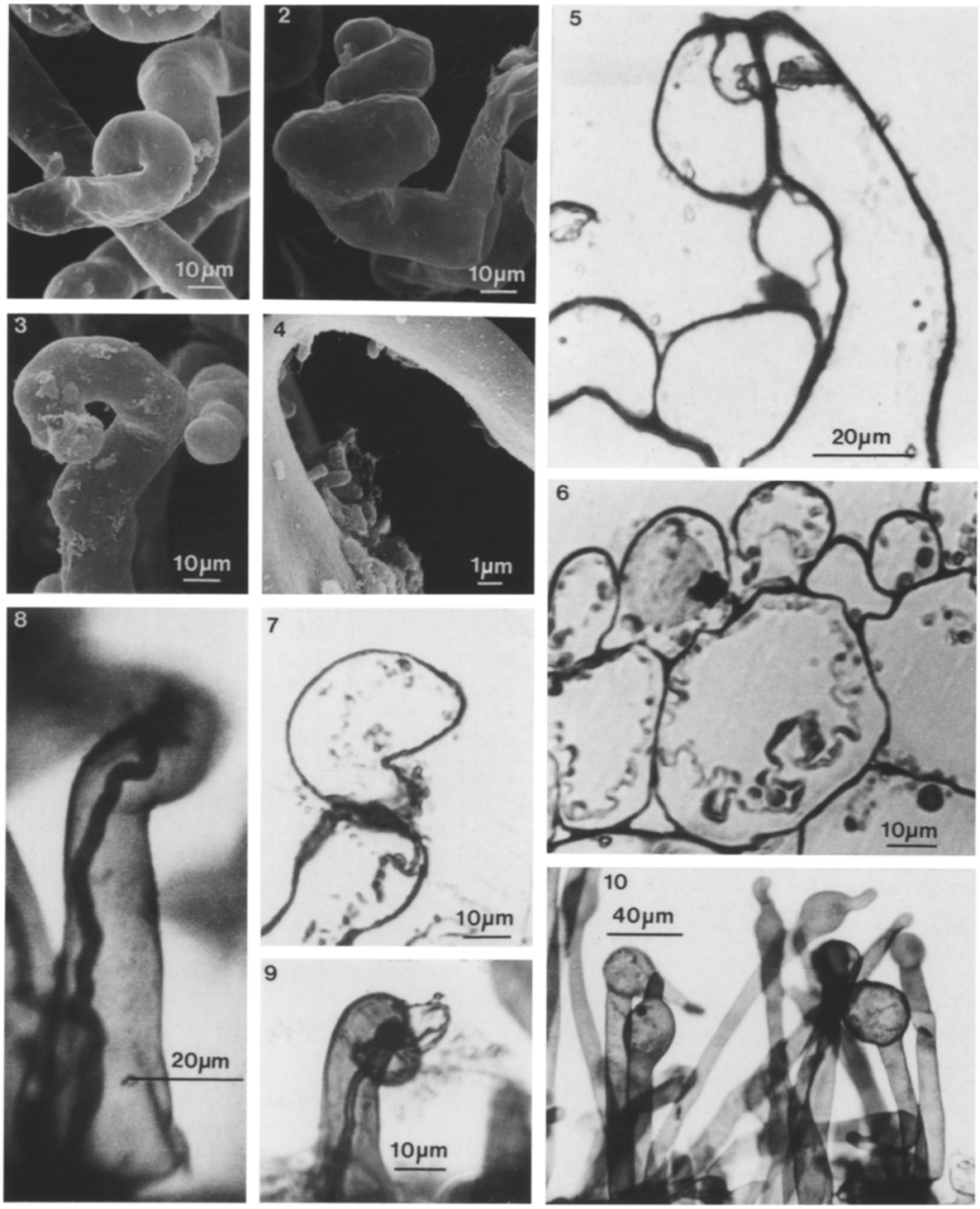
Methylene-Blue staining for detecting infection threads. The method of Vasse and Truchet (1984) was used to examine infection threads within root hairs. Fixed roots were rinsed in buffer (3 × 1 h) and dehydrated in an ethanol-water series. After dehydration, specimens were cleared by immersion for 2 h in 99% solution of methyl benzoate, washed again with absolute ethanol (3 × 1 h), progressively rehydrated, and finally stained in 0.01% (w/v) aqueous Methylene Blue.

Results

Observations using scanning electron microscopy (SEM)

Preliminary examination of root segments was made under a dissecting microscope. The following treatments were selected for detailed SEM examination: main roots 100 mol·m^{-3} NaCl, 200 mol·m^{-3} PEG, days 1–6 inclusively; 50 mol·m^{-3} NaCl, 100 mol·m^{-3} PEG, day 3; lateral roots 100 mol·m^{-3} NaCl, 200 mol·m^{-3} PEG, day 3.

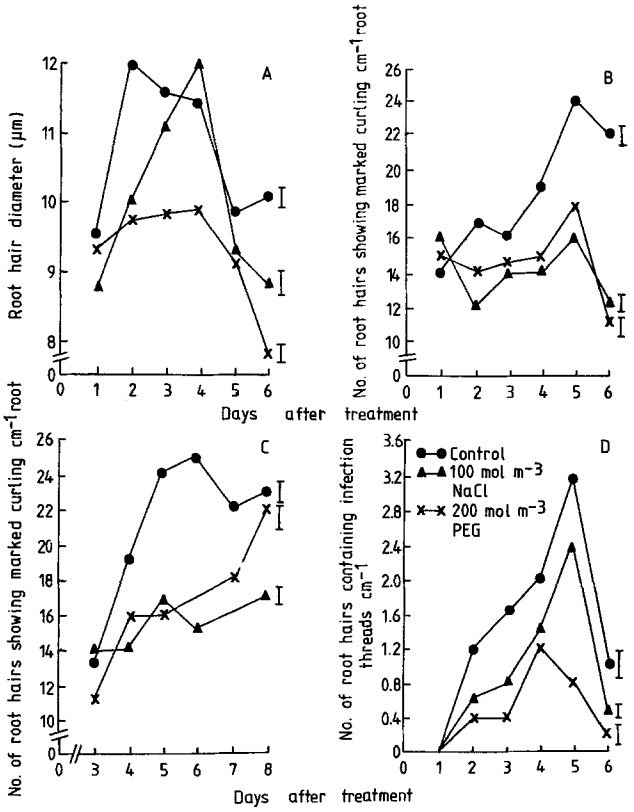
Bacterial colonization and curling. No clear effects of NaCl or PEG were seen on bacterial colonization of root hairs at magnifications ranging from × 6 000 to × 15 000 although PEG-treated plants appeared to have fewer adhering rhizobia. Bacteria were seen in random orientation with respect to the hair surface, attached in a polar manner without microfibrils and associated with an extensive system of microfibrils (Fig. 4). Thus all stages in



Figs. 1–4. Scanning electron micrographs of main root hairs of *Vicia faba*. **Figs. 1, 2.** Helical type of root-hair deformation 72 h after inoculation and 50 mol·m^{-3} NaCl treatment (**Fig. 1**) and 96 h after inoculation and treatment with 100 mol·m^{-3} NaCl (**Fig. 2**). **Fig. 3.** Marked curling and bacterial colonization near the root-hair tip 48 h after inoculation and treatment with PEG 200 mol·m^{-3} **Fig. 4.** Bacteria in the crook adhering to the inside walls in lateral and polar orientation in association with microfibrils 72 h after inoculation and treatment with 200 mol·m^{-3} PEG. **Figs. 1–3**, ×1000; **Fig. 4**, ×6600

Figs. 5–7. Light micrographs of *V. faba* roots ultramicrotome sections; 4 μm thickness), showing the structure of main root hairs, epidermal and hypodermal cells 5 d from inoculation and beginning of stress treatment. **Fig. 5.** No signs of plasmolysis in root-hair and epidermal cells of the control plant; ×828. Some of the root hairs as well as epidermal and hypodermal cells plasmolysed and their membranes folded under the treatments of NaCl 100 mol·m^{-3} (**Fig. 6**; ×785) and PEG 200 mol·m^{-3} (**Fig. 7**; ×747)

Figs. 8–10. Light micrographs of fixed and Methylene-Blue-stained *V. faba* root hairs. **Figs. 8, 9.** Lateral root hairs showing marked curling and containing infection threads. **Fig. 8.** Control root hair; ×882. **Fig. 9.** NaCl 100 mol·m^{-3}; ×853. **Fig. 10.** Swollen-tipped root hairs after treatment with NaCl 100 mol·m^{-3}; ×311



Figs. 11 A–D. Effects of NaCl (100 mol·m^{-3}) and PEG (200 mol·m^{-3}) on root hair expansion (**A**), curling of main (**B**), and lateral (**C**) root hairs and infection-thread formation (**D**) in *V. faba*. **A** Main root-hair diameter varied significantly ($P<0.01$) both with NaCl and PEG treatments and age. **B, C** Curling in main and lateral root was affected significantly ($P<0.001$) by both NaCl and PEG treatment and age. **D** Both NaCl and PEG treatments and age affected infection thread number significantly ($P<0.001$) in main root hairs. *Bars* represent SDs, $n=5$

the pre-infection process as proposed by Dazzo et al. (1984) for the clover-*Rhizobium trifolii* symbiosis were present.

Deformation, curling and shepherd's crook formation, characteristic of early phase of root-hair infection, were seen under both NaCl and PEG treatments (Figs. 1–3), but were less frequent in PEG-stressed roots. The helical deformations first seen with clover root hairs in the presence of living rhizobia (Ervin and Hubbell 1985) are seen in Figs. 1 and 2.

Diameter of root hairs. Apparent shrinkage of some of the main root hairs was seen as a result of NaCl (100 mol·m^{-3}) and PEG (100 and 200 mol·m^{-3}) treatments; it was more frequent with (200 mol·m^{-3}) PEG. This effect was examined further by measuring (under a dissecting microscope) root-hair diameter in SEM photographs taken at magnifications in the region of ×200 and ×1000. The diameters of main root hairs (Fig. 11 A) varied significantly ($P<0.01$) both with NaCl and PEG treatments and age; there was no significant stress × age interaction. The effect of PEG was approximately double that of NaCl. In control roots, hair diameter reach a maximum and then declined after 6 d of treatment possibly because of the senescence of root hairs. Treatment with PEG prevented hairs from reaching the same maximum diameter but some decline was still observed. NaCl delayed the time at which maximum expansion was observed. The diameter of lateral root hairs was not significantly affected by NaCl and PEG treatments (being reduced by about 10%), possibly because lateral root hairs were very young and developed after the commencement of the treatment (data not shown).

Observations using light microscopy

Root-hair formation. Sections of main and lateral roots (1–4 μm thickness) were examined after 5 d of treatment. Both NaCl and PEG reduced root-hair production on the main roots by about 24% and 30% respectively, the effects being just significant ($P<0.05$) (data not shown).

Root-hair structure. The structure of some of the main root hairs, epidermal and hypodermal cells, was distorted as a result of NaCl (100 mol·m^{-3}) and PEG (200 mol·m^{-3}) treatments; cells were plasmolysed and their membranes folded (Figs. 6, 7).

Hair curling and formation of infection threads in fixed and stained roots. Both NaCl (100 mol·m^{-3}) and PEG (200 mol·m^{-3}) treatments reduced the proportion of markedly curled hairs on main and lateral roots (Fig. 11 B, C). The proportion of main root hairs containing infection threads was significantly ($P<0.001$) reduced under NaCl (38%) and PEG (67%) (Fig. 11 D); in lateral root hairs it was reduced by about 18% under both NaCl and PEG but the effects were not statistically significant (data not shown). Infection threads were detected only in markedly curled root hairs regardless of whether they were short or long (Figs. 8, 9). Some of the root hairs had swollen tips; these occurred under all treatments, but were most frequent with NaCl (Fig. 10). None of them contained infection threads.

Effects of NaCl and PEG on nodulation

Nodule number. As many of the rhizobial infections abort inside root hairs and relatively few infections

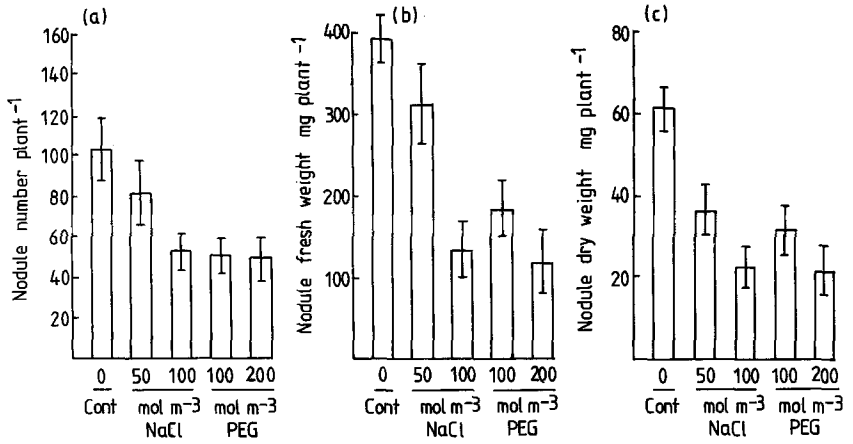


Fig. 12a–c. Effects of NaCl (50 and 100 mol·m^{-3}) and PEG (100 and 200 mol·m^{-3}) on nodule number (**a**), nodule fresh weight (**b**) and nodule dry weight (**c**) after three weeks of treatment. *Bars* represent SDs, $n=6$

develop into nodules (Calvert et al. 1984), nodulation measured as nodule number is usually considered an indication of fully successful infections. After three weeks of treatments, NaCl (100 mol·m^{-3}) and PEG inhibited nodulation by about 50% (Fig. 12a).

Nodule weight. The fresh weight of nodules per plant was reduced by about 70% under NaCl (100 mol·m^{-3}) and PEG (200 mol·m^{-3}); dry weights were reduced similarly (Fig. 12b, c). This is more than would be predicted on the basis of nodule number and reflects the fact that nodule size (weight per nodule) was also reduced.

Discussion

A favourable rhizosphere environment is of vital importance to the root hair-*Rhizobium* interaction, as it is not only encourages the growth and multiplication of rhizobia but also ensures the healthy development of root hairs. The former increases the inoculum potential of rhizobia and the latter provides ample infection sites (Tu 1981). Therefore, conditions that favour root-hair development, and rhizobial growth and multiplication are generally favourable for nodulation.

Sodium chloride and PEG had no obvious effect on bacterial colonization of root hairs of *V. faba* plants. Similar results have been found with soybean where rhizobial colonies of inoculated root surfaces were not affected by 80 mol·m^{-3} NaCl (Singleton and Bohlool 1984). Overall, the literature is consistent on two points: first, that there is considerable strain variability in survival and growth of rhizobia under saline conditions, and second, that rhizobia are generally more able to cope with salinity than their host legumes (Sprent 1984). For water stress, Worrall and Roughley (1976) found that a reduction of soil moisture from 5.5 to 3.5% completely inhibited nodulation in *Trifolium subterraneum* without affecting rhizobial numbers in the rhizosphere, and Williams and Mallorca (1984) working with soybean, found that 6% PEG (MW 4000) had no effect on the growth of *R. japonicum* in YM broth medium. The reduction in nodule numbers observed was not associated with fewer rhizobia in the rhizosphere of stressed plants.

In the present study, root-hair formation was not greatly affected, but marked curling was reduced in main and lateral root hairs (Fig. 11 B, C). This differs from the results of Lakshmi-Kumari et al. (1974) who found a reduction in both root hair number and curling in NaCl-treated *Medicago sativa*. Further, it is shown here that expansion of root hairs was slowed down by NaCl and inhibited by PEG treatment (Fig. 11 A). This might be due to effects of NaCl and PEG on root-hair cell turgor and/or wall structure. Solutions of PEG with various osmotic potentials (0.6–1.0 MPa) reduced root diameter in *V. faba* (Rowse and Goodman 1981). Reduction of turgor pressure inhibits cell-wall growth in root hair of *Tradescantia fluminensis* (Schroter and Sievers 1971), and in wheat seedlings, hair diameter was 20% greater in distilled water than in the nutrient solution (Ekdahl 1957).

Root hairs with swollen tips were most common under NaCl treatment (Fig. 10) and were never seen to contain infection threads. Thus, although this morphology was shown by Ervin and Hubbell (1985) to be one of the deformations characteristic of *T. repens* root hairs after inoculation with *R. trifolii*, it may not be correlated with infection. Further, swollen-tipped root hairs have also been seen with non-legumes (e.g. wheat, Ekdahl 1953) as a result of osmotic stress. Root hairs of

V. faba plants grown under NaCl and PEG treatments showed plasmolysis and their membranes folded. This effect, which is extended to some of the epidermal and hypodermal cells (Figs. 6, 7), would be an obstacle to the initiation and development of infection threads.

Plasmolysis of root hairs under osmotic stress has been reported in wheat (Ekdahl 1953). Structural damage due to PEG treatment in maize root epidermis (Clamporova 1981) and ultrastructural changes in wheat and *V. faba* have been found under both NaCl and PEG treatments (Udovenko et al. 1970).

Both the inhibition of hair expansion and the decrease in the proportion of root hairs containing infection threads (30% under NaCl and 52% under PEG) could be related to calcium nutrition, which is known to be associated with infection of root hairs in *T. repens* (Sethi and Reporter 1981). Salinity reduces both uptake and intercellular concentrations of calcium in cotton roots (Gerard 1971; Gerard and Hinojosa 1973) and lowers the concentration of cell-wall cations (Ca^{++}, Mg^{++}, K^{+} and Na^{+}) of *Phaseolus vulgaris* roots (Bigot et al. 1983). The shape and elongation of root hairs are highly dependent on the concentration of the calcium ions in the surrounding medium, and hardening of the apical hair walls takes place through a gradual formation of calcium pectates (Cormack 1949). Apical infection, which is common in leguminous root hairs (e.g. soybean, Calvert et al. 1984 and clover, Bhuvaneswari and Solheim 1985) is also the usual mode of infection in the *R. leguminosarum-V. faba* symbiosis (Figs. 8, 9). The inhibition of the growth of apical walls and hardening of the root hairs through the effects of NaCl and PEG on calcium uptake might be an explanation of the reduction in root-hair curling and also formation of swollen hair tips in *V. faba* plants.

Consistent with this suggestion, we have data (not shown) indicating that calcium can improve infection and nodulation in control, NaCl and PEG treatment plants, an observation confirming the work of Yousef (1982).

In this investigation with *V. faba*, 100 $mol \cdot m^{-3}$ NaCl and 200 $mol \cdot m^{-3}$ PEG inhibited nodule number by about 50% and nodule weight by more than 60% (Fig. 12). This is a smaller effect than found for soybean, which showed about 90% inhibition of nodulation with only 80 $mol \cdot m^{-3}$ NaCl (Singleton and Bohlool 1984); 200 $mol \cdot m^{-3}$ NaCl was completely inhibitory (Tu 1981). Hairs of roots of plants like soybean appear to have only one infectible period, whereas others such as clover, have two, one early and one later in development (Bhuvaneswari et al. 1981). Since taxonomically and in its nodule structure and function, *V. faba* is more closely related to clover than to soybean (Sprent 1980), it is likely to have two infectible periods: this may partly explain why its nodulation is more resistant to NaCl and PEG treatment than that of soybean.

The results presented here indicate that in *V. faba*, PEG affects both infection and nodule development whereas the major effect of NaCl is on nodulation rather than infection.

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