Symbiosis, 6 (1988) 81–96
Balaban Publishers, Philadelphia/Rehovot

Infection and Root Nodule Structure in the *Rhizobium galegae* sp. nov.-*Galega* sp. Symbiosis

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Received April 28, 1988; Accepted May 24, 1988

Abstract

The specificity of the *Rhizobium galegae* sp. nov.–*Galega* sp. interaction was studied microscopically. *R. galegae* strains 1261R and B7i deformed root hairs of both *G. orientalis* and *G. officinalis*. Infection threads started from cauliflower-like structures on long root hairs or from very short deformed root hairs. *R. galegae* 1261R and B7i did not induce any changes in the root hairs of *Medicago sativa* or *Lotus corniculatus*. These strains deformed root hairs of *Trifolium pratense*, but no infection threads were seen. *R. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* ANU 843, and *R. loti* NZP 2213 did not induce any changes in the root hairs of *Galega* plants. The structure of both effective and ineffective *G. orientalis* root nodules induced by *R. galegae* strains was indeterminate. The root nodules had an apical meristem, an infection zone, and a central zone containing cells filled with bacteroids.

Keywords: *Rhizobium galegae*, *Galega*, host-specificity, infection, root nodule structure

1. Introduction

Goat's rue (*Galega orientalis*) is a promising forage legume suitable for low temperature and acid soil conditions in Finland (Varis, 1986). It is able to fix atmospheric nitrogen when living in symbiosis with root nodule bacteria. *Rhizobium* strains nodulating *G. orientalis* and *G. officinalis* form a unique group among fast-growing rhizobia. The *Galega* rhizobia are very host specific. *Rhizobium* strains isolated from *G. orientalis* only form an effective symbiosis with *G. orientalis* and an ineffective symbiosis with *G. officinalis*. For strains isolated from *G. officinalis* the situation is reversed (Lindström et al., 1983). Cross-nodulation results, numerical taxonomy, maximum growth temperatures, phage-typing, DNA-homology, ribosomal RNA homology, and lipopolysaccharide and whole cell protein patterns distinguish the *Galega* rhizobia from other root nodule bacteria (Jarvis et al., 1986, Lindström et al., 1983, Lindström and Lehtomäki, 1988, Lipsanen and Lindström, 1986; Lipsanen and Lindström, 1988; Wedlock and Jarvis, 1986). Based on these results a new species, *Rhizobium galegae* sp. nov., will be proposed.

Root nodule bacteria can infect their host plants in different ways. Rhizobia enter several temperate crop and forage legumes, including clovers, alfalfa, peas, and some beans by first deforming root hairs of the host plant and then penetrating the root cortex cells via infection threads, starting from deformed root hairs (Rolfe and Shine, 1984). Many tropical legumes, including *Arachis*, *Stylosanthes*, many mimosoid legumes, the stem nodulating *Aeschynomene* and *Sesbania*, as well as the non-legume *Parasponia*, are infected differently. Rhizobia infect directly through the epidermis or through surface breaks, caused, for example, by lateral root emergence. In this infection type the initial passage of rhizobia is intercellular. Infection threads may never form (*Arachis* and *Stylosanthes*), or may form at a later stage of development (*Parasponia* and possibly many mimosoid legumes) (Sprent et al., 1987).

There are two basic types of legume root nodules. Indeterminate root nodules are typical of temperate legumes such as clovers, peas, medics and beans. These nodules have a persistent meristem and zones containing bacteria in different stages of differentiation. Plant cells in the symbiotic zone are filled with bacteroids having nitrogenase activity. The bacteroids of the senescence zone have started to deteriorate. Determinate root nodules are found in tropical legumes, *Phaseolus, Vigna* and soybean. Their meristem is non-persistent and the bacteria are in approximately the same stage of development (Rolfe and Shine, 1984).

We studied the specifity of the *R. galegae-Galega* interaction in greater detail by observing root hair deformation and infection thread formation in combinations of *G. orientalis* and *G. officinalis* and other fast-growing rhizobia (*R. meliloti*, *R. leguminosarum* bv. *trifolii*, *R. loti*), as well as in combinations of *R. galegae* strains and the host plants of the other rhizobia tested (*Medicago sativa*, *Trifolium pratense*, *Lotus corniculatus*). We also studied the nodule structure in an effective symbiosis between *G. orientalis* and its homologous *R. galegae* strain, and in an ineffective symbiosis between *G. orientalis* and a *R. galegae* strain isolated from *G. officinalis*.

2. Materials and Methods

Bacteria

Bacterial strains are shown in Table 1. The strains are maintained in the culture collection of the Department of Microbiology, University of Helsinki (HAMBI).

Table 1. Bacterial strains

Bacterial strain	Host plant
Rhizobium galegae	
1261R (HAMBI 1174)	*Galega orientalis*
B7i (HAMBI 490)	*G. officinalis*
R. meliloti	
Rm1021	*Medicago sativa*
R. leguminosarum bv. *trifolii*	
ANU 843 (HAMBI 1342)	*Trifolium subterraneum*
R. loti	
NZP 2213 (HAMBI 1129)	*Lotus tenuis*

Plant material

Galega orientalis (goat's rue) unbred seeds were from Viikki Experimental Farm, Helsinki, Finland. *G. officinalis* seeds were collected from wild plants and were a gift from Paul Buckley, Massey University, Palmerston North, New Zealand. *Medicago sativa* (alfalfa) cv. Iroquois was a gift from Fred Ausubel, Massachusetts General Hospital, Boston, MA, USA, *Trifolium pratense* (red clover) cv. was Hankkija's Venla from Finland, and *Lotus corniculatus* seeds were a gift from the Department of Plant Husbandry, University of Helsinki, Finland.

Plant tests

Plant tests were done according to Truchet et al. (1984) with some modifications. Seeds were first rinsed with 70% ethanol for 30 sec and then with sterile water 3 × 10 min. They were then sterilized with 0.1% $HgCl_2$ for 5 min and rinsed with sterile water 6 × 10 min. Sterilized seeds were germinated on YEM-Congo red agar plates (Vincent, 1970) in the dark at room temperature until the roots were about 1 cm long. Then the seedlings were transferred onto Jensen-agar slants (Vincent, 1970), one plant per test tube (2 cm diameter×15 cm height). Plants were grown in a growth chamber at 22°C with a 16 hr light and an 8 hr dark period. Plants were inoculated after 5 days. Inoculant strains were grown for 2 days on YEM-Congo red plates at 28°C, and bacteria were suspended in sterile water to a final concentration of 10^8/ml. Portions (0.5 ml) of this suspension were added onto each slant, the root was flushed five times with the suspension with a Pasteur pipette, and the suspension was removed.

Microscopy of root hairs

Roots of at least 30 plants from every *Rhizobium*-legume combination were studied. The root hairs were stained according to Vasse and Truchet (1984) with a 0.01% methylene blue solution, and observed under bright field microscopy 3,7,11, and 14 days after inoculation.

Microscopy of root nodules

Five effective and five ineffective root nodules of *G. orientalis* inoculated with *R. galegae* strains 1261R and B7i, respectively, were studied microscopically. Samples for light and electron microscopy were prepared according to a modification of the method of Truchet et al. (1984). Root nodules were fixed 2 × 1 hr with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), post-fixed for 1.5 hr with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2), dehydrated in an ethanol series and embedded in Epon (LADD). For light microscopy, sections of 1 μm were stained with basic fuchsin (G.T. Gurr Ltd.) and methylene blue (Merck) according to Huber et al. (1968). For electron microscopy, thin sections of 0.1 μm were stained with uranyl acetate for 30 min and lead citrate for 1–2 min. The thin sections were examined with a JEOL 100 CX electron microscope with acceleration voltage of 80 kV.

Table 2. Cross-inoculation results

Bacterial strain	Plant														
	Galega orientalis			*Galega officinalis*			*Medicago sativa*			*Trifolium pratense*			*Lotus corniculatus*		
	D	IT	N	D	IT	N	D	IT	N	D	IT	N	D	IT	N
Rhizobium galegae															
1261R	+	+	+	+	+	(+)	–	–	–	+	–	–	–	–	–
B7i	+	+	(+)	+	+	+	–	–	–	+	–	–	–	–	–
R. meliloti															
Rm1021	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–
R. leguminosarum															
bv. *trifolii*															
ANU 843	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–
R. loti															
NZP 2213	–	–	–	–	–	–	–	–	–	NT	NT	NT	+	+	+

D = deformation of root hairs

IT = infection thread formation

N = nodulation

+ = reaction

– = no reaction

(+) = ineffective nodules

NT = not tested

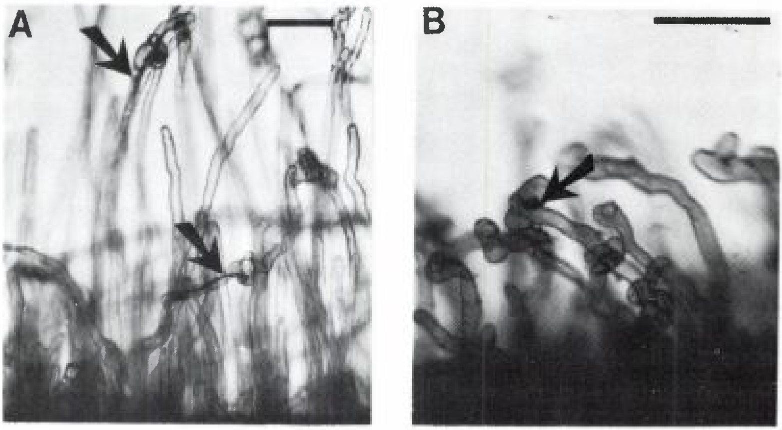


Figure 1. Root hairs of *G. orientalis* inoculated with (A) *R. galegae* 1261R, (B) *R. galegae* B7i. The arrow points at an infection thread. Scale bar = 100 μm.

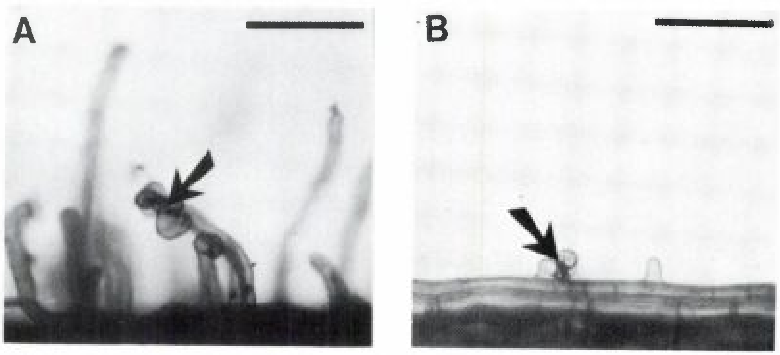
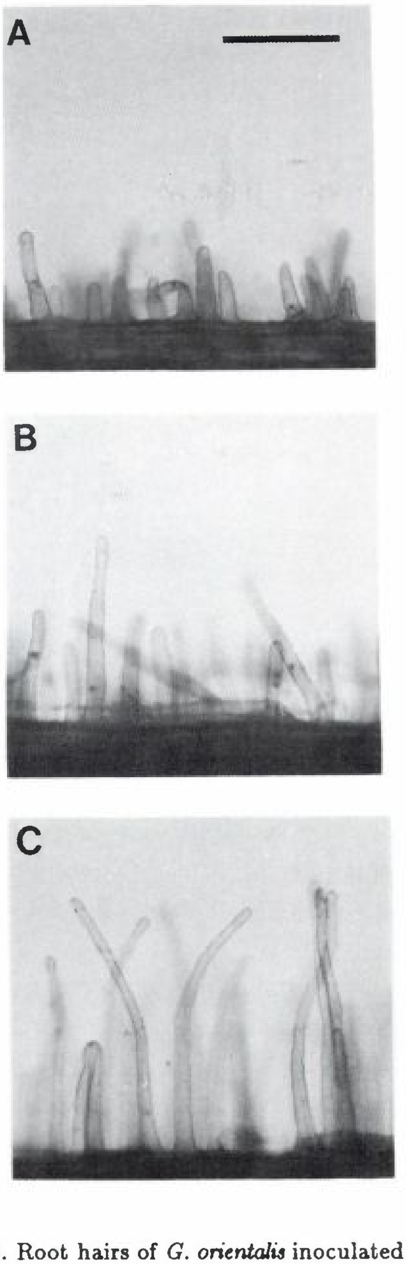


Figure 2. Root hairs of *G. officinalis* inoculated with (A) *R. galegae* B7i, (B) *R. galegae* 1261R. The arrow points at an infection thread. Scale bar = 100 μm.

3. Results

Results from cross-inoculation experiments are shown in Table 2. *R. galegae* strains deformed root hairs of both *G. orientalis* and *G. officinalis* plants to cauliflower-like structures. Infection threads were seen in very short root hairs or starting from cauliflower-like structures of long root hairs (Figs. 1 and 2). The tested *R. meliloti*, *R. leguminosarum* bv. *trifolii*, and *R. loti* strains did not induce any changes in the root hairs of the *Galega* plants (Figs. 3 and 4). *R. galegae* strains did not deform or form infection threads in the root hairs of *M. sativa* or *L. corniculatus*. However, they did deform the root hairs of *T. pratense*, but no infection threads were seen (Figs. 5 and 6).



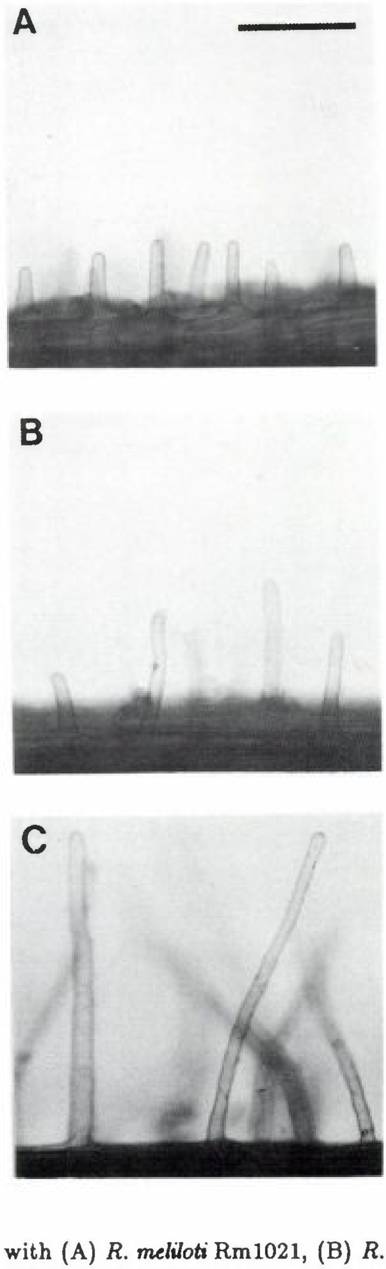
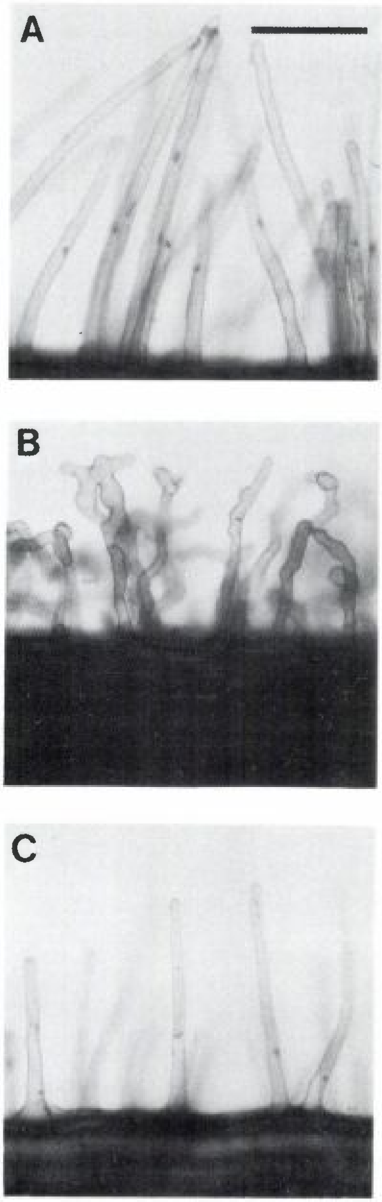


Figure 3. Root hairs of *G. orientalis* inoculated with (A) *R. meliloti* Rm1021, (B) *R. leguminosarum* bv. *trifolii* ANU 843, (C) *R. loti* NZP 2213. Scale bar = 100 μm.

Figure 4. Root hairs of *G. officinalis* inoculated with (A) *R. meliloti* Rm1021, (B) *R. leguminosarum* bv. *trifolii* ANU 843, (C) *R. loti* NZP 2213. Scale bar = 100 μm.



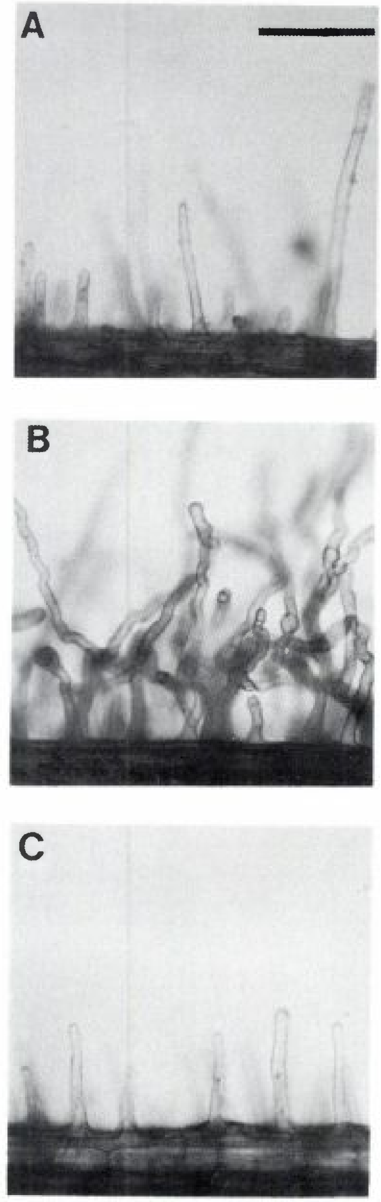


Figure 5. Root hairs inoculated with *R. galegae* 1261R (A) *M. sativa*, (B) *T. pratense*, (C) *L. corniculatus*. Scale bar = 100 μm.

Figure 6. Root hairs inoculated with *R. galegae* B7i (A) *M. sativa*, (B) *T. pratense*, (C) *L. corniculatus*. Scale bar = 100 μm.

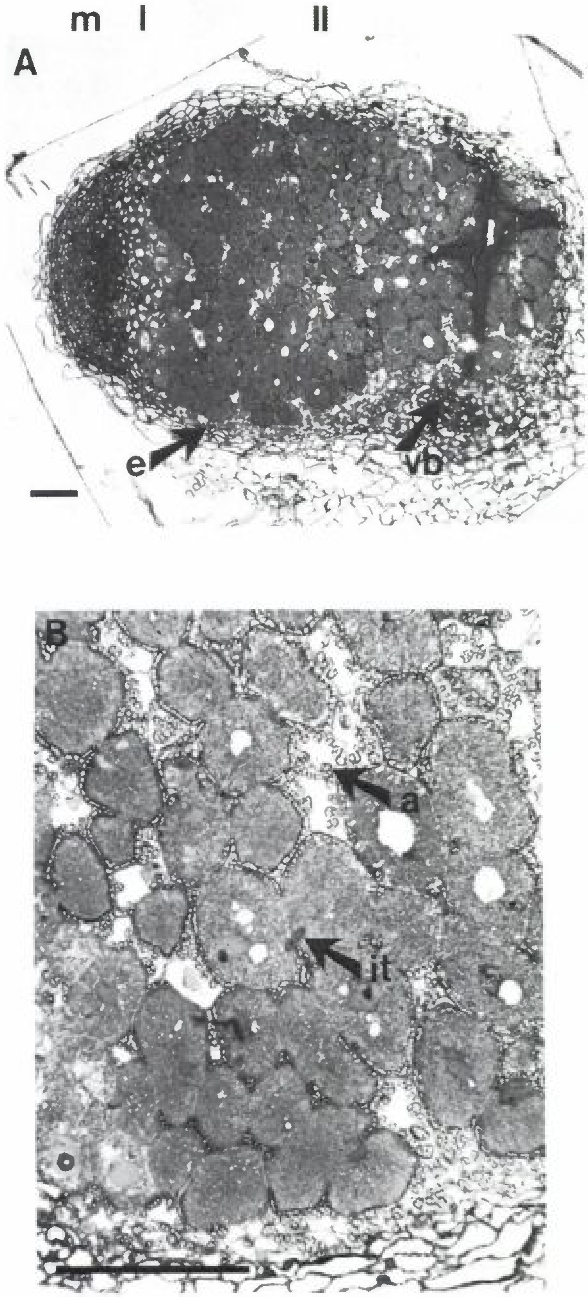


Figure 7. Semithin sections of an effective root nodule of *G. orientalis* inoculated with *R. galegae* 1261R. (A) m = meristem, I = infection zone, II = central zone, e = endodermis, vb = vascular bundle. (B) it = infection thread, a = amyloplast. Scale bar = 100 μm.

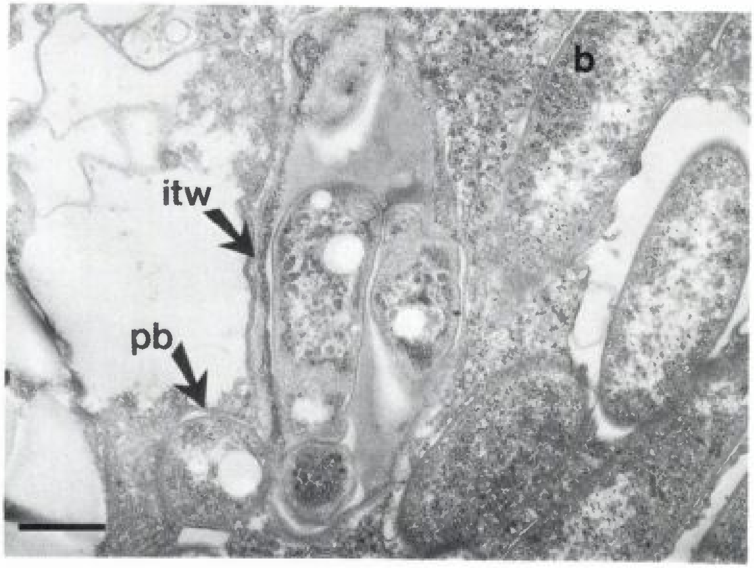


Figure 8. Thin section of an effective root nodule of *G. orientalis* inoculated with *R. galegae* 1261R. b = bacteroid, pb = peribacteroid membrane, itw = infection thread wall. Scale bar = 1 μm.

Both effective (Fig. 7) and ineffective (Fig. 9) *G. orientalis* root nodules, had the same basic structure: an apical meristem, an infection zone, a central zone with cells filled with bacteroids, an endodermis, and vascular bundles. Slight differences between the effective and ineffective nodules could, however, be observed. The ineffective nodules contained more noninfected plant cells than the effective nodules. In these cells there were no bacteria but large amounts of starch granules.

Both effective and ineffective nodules had infection threads in nodule cells (Figs. 7, 8, 9, and 10), and each bacteroid was surrounded by its own peribacteroid membrane (Figs. 8 and 10). The bacteroids in the ineffective nodules seemed to contain more granules, probably storage material, than the bacteroids in the effective nodules.

4. Discussion

R. galegae strains infect their host plants by deforming root hairs and penetrating the root cortex cells via infection threads starting from deformed root hairs. The same way of infection has been observed for many other fast-growing *Rhizobium* species (*R. meliloti*, *R. leguminosarum*) living in symbio-

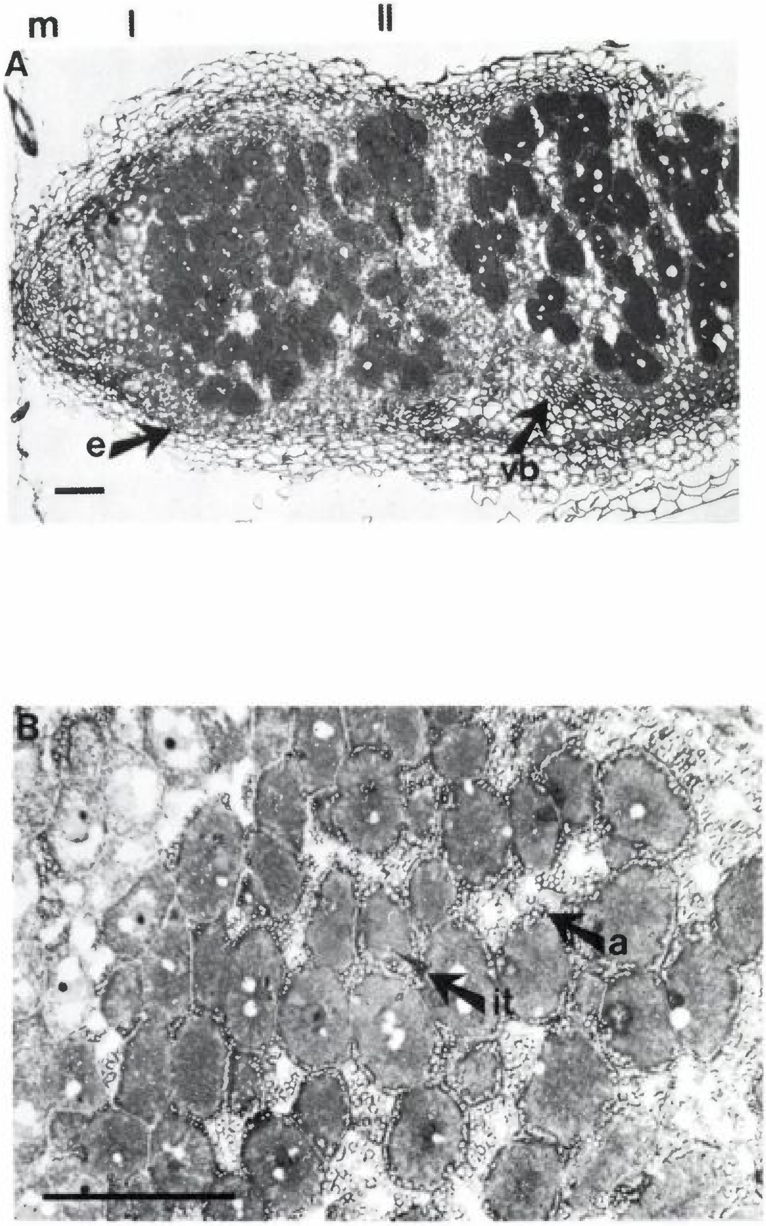


Figure 9. Semithin section of an ineffective root nodule of *G. orientalis* inoculated with *R. galegae* B7i, (A) m = meristem, I = infection zone, II = central zone, e = endodermis, vb = vascular bundle. (B) it = infection thread, a = amyloplast. Scale bar = 100 μm.

Figure 10. Thin section of an ineffective root nodule of *G. orientalis* inoculated with *R. galegae* B7i. b = bacteroid, pb = peribacteroid membrane, itw = infection thread wall, g = granules. Scale bar = 1 μm.

sis with temperate legumes (alfalfa, peas, clovers) (Rolfe and Shine, 1984). The structure of the root nodule of goat's rue is indeterminate. The other temperate legumes mentioned have the same nodule type (Rolfe and Shine, 1984). Although the mode of infection and the basic nodule structure are the same as in many other *Rhizobium*-legume combinations, the *Rhizobium galegae* — *Galega* interaction seems to be very specific.

Other fast-growing rhizobia tested (*R. meliloti*, *R. leguminosarum* bv. *trifolii*, and *R. loti*) did not induce any visible reaction in the roots of *Galega* plants. *R. galegae* strains did not cause any changes in the roots of alfalfa or lotus. *R. galegae* strains deformed root hairs of red clover but no infection threads were seen. *R. meliloti* caused the same reaction on red clover in our experiment. Yao and Vincent (1969) defined three categories of root hair deformation (1) branched, (2) moderately curled, and (3) markedly curled — having the tip curled at least 360°. They studied deformation of root hairs of *M. sativa*, *Trifolium glomeratum*, and *Phaseolus atropurpureus* (siratro) inoculated with rhizobia isolated from the root zone of these plants. The markedly curled condition was practically restricted to the host plant associated with virulent homologous rhizobia. Branching and moderate curling were generally most frequent with homologous associations but were found in some cases with most of the rhizobia tested on *T. glomeratum*, including avirulent *R. trifolii*. Our results, including the moderate curling of *T. pratense* inoculated with *R. galegae* or *R. meliloti*, are in agreement with the results of Yao and Vincent (1969) on the specificity of marked curling and with the reports reviewed by Dart (1977).

R. galegae strains isolated from *G. orientalis* form effective, nitrogen fixing root nodules on *G. orientalis*. *R. galegae* strains isolated from *G. officinalis* form ineffective root nodules on *G. orientalis*,which do not show nitrogenase activity (Lindström et al., 1983). Both effective and ineffective *G. orientalis* root nodules have an apical meristem, infection zone, a central zone containing plant cells filled with bacteroids, and peripheral vascular bundles. The bacteroids are enclosed in a peribacteroid membrane, one bacteroid in each membrane envelope. There were only slight differences between effective and ineffective *G. orientalis* root nodules. The central zone of the effective nodule contained fewer noninfected plant cells than the central zone of the ineffective nodule. The bacteroids in the effective nodule contained less granules than the bacteroids in the ineffective nodule. Based on these findings it is not possible to explain why the ineffective *G. orientalis* root nodules do not fix nitrogen. The structure of the root nodules must be studied in greater detail to find out, at which stage the development of the symbiosis ceases. Wilson et al. (1987) suggested that host specific nitrogen fixation could be similar to host specific nodulation in that a specific negative interaction occurs at an early stage in the nodulation pathway, but that the barrier lies in nodule invasion rather than nodule induction. The defects could lie in specific signalling between the symbiotic partners or in some aspect of nodule physiology. Also, the different host plants could supply different carbon substrates to the bacteroids, and ineffective strains might be deficient in the utilization of host specific carbon sources. Reduction in the levels of exported, fixed nitrogen can lead to a failure by the plant to induce the enzymes necessary for nitrogen assimilation and to early senescence of the nodules. Hrabak et al. (1985) reported that the structure of ineffective root nodules formed by *R. leguminosarum* on *Trifolium subterraneum* was quite similar to that of effective nodules. However, a unique feature was a rapidly advancing zone of senescence proceeding toward the meristematic end of the nodule between 30 and 40 days after inoculation. Fusions of peribacteroid membranes occurred and many lysosome-like organelles were present in degenerating host cells. Normal early development of nodules, followed by lack of persistence of the bacteroid state, is a common feature of ineffective nodules from many legumes. Hrabak et al. (1985) suggested that insufficient leghaemoglobin production could cause a decreased oxygen flux into the nodule, and that the resulting oxygen limitation of the bacteroid tissue prevents nitrogen fixation and accelerates senescence. In the early stages of nodule differentiation, control of the oxygen level might also be necessary for nitrogenase induction (Sprent et al., 1987). The hypotheses for the development of the ineffective *Rhizobium galegae-Galega* symbiosis still need to be verified in future experiments.

Acknowledgements

We are grateful to G. Truchet and J. Vasse for instructing and guiding us in the field of root hair and nodule microscopy.

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