

Review article

***Rhizobium* Nodulation of Non-Legumes**

R.W. RIDGE¹, B.G. ROLFE¹, Y. JING² and E.C. COCKING³

¹*The Australian National University, Research School of Biological Sciences
GPO Box 475, Canberra ACT 2601 Australia*

Tel. 61 6 249 4054, Fax 61 6 249 0754

²*Institute of Botany, Academia Sinica, 141 Xiwai Dajie, Beijing 100044, China*
Fax 86 183 12840

³*Plant Genetic Manipulation Group, Department of Botany
University of Nottingham NG7 2RD, UK*

Fax 44 602 420 825

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Abstract

As symbiotic systems are the most ecologically sound and the cheapest source of fixed nitrogen, agricultural scientists have long wished to extend the host range of rhizobia, with their excellent nitrogen-fixing ability, to the cereals rice and wheat. Recent developments in cell biology techniques to remove the cell wall at the apices of root hairs of a wide range of plants, both legumes and non-legumes, as well as advances in *Rhizobium* genetics, have opened up new approaches to the infection of cereals. Furthermore, the isolation and construction of new *Rhizobium* strains which can induce nodule-like structures on the roots of different rice varieties has re-stimulated interest in this area of agricultural research. The nodule-like structures formed on the roots of rice seedlings are most probably a form of modified lateral root and vary enormously in their size and internal structure. The best examples have vascular bundles associated with these root outgrowths, and inter- and intra-cellularly located bacteria. The infected plant cells appeared to be unstressed and the intracellular bacteria 'packaged' within membranes. As yet, there is little hard evidence of infection thread formation or precisely how these structures may be induced. Because the frequency of formation of these 'nodules' is so variable, progress in this research area is slow, but there are now sufficient observations to establish that a real phenomenon exists, and it should therefore become a field of research in biological nitrogen fixation.

Keywords: *Bradyrhizobium*, nodules, non-legumes, *Parasponia*, *Rhizobium*

1. Introduction

The benefits to agriculture from the legume symbiosis with *Rhizobium* have been widely recognized throughout history, and the international farming community has inoculated legumes with various strains of rhizobia since early this century. Agricultural scientists have long held the wish to be able to transfer the nitrogen-fixing ability of *Rhizobium* to other plant groups, especially to the monocotyledons. The introduction of a similar bacterial seed inoculant system for rice would be an ecologically sound and cheap supplement for nitrogenous fertilizer. Over the last few years, a number of groups have been attempting to extend the *Rhizobium*-legume symbiosis to non-legumes by applying various strains and conditions to induce nodule-like structures, with varying degrees of success. We summarise the current knowledge in the field, as well as unpublished results from our laboratories.

Despite the encouraging results from some laboratories, we cannot overstate the importance of understanding the complex cytological and biochemical events that underlie the development of the symbiosis. The considerable work on *Rhizobium* and *Bradyrhizobium-Parasponia* symbioses, for example (set out below) is an attempt at understanding these principles through cellular and molecular biology. It is clear from work on the other non-legumes that the key to success may be the analysis, understanding and manipulation of the regulation that controls host specificity. If there are to be working symbioses with non-legumes, especially the monocotyledons, then a thorough working knowledge of the system is paramount for further manipulation and improvement of the crop.

2. *Parasponia andersonii* nodulation

In symbiotic associations between soil bacteria (*Rhizobium* and *Bradyrhizobium*) and the members of the plant family Leguminosae, the rhizobia infect the root via either the root hairs or by crack entry (by entering the plant through the small areas of damaged cells caused by emerging laterals or adventitious roots) and induce the formation of morphologically-defined structures called nodules (Rolfe and Gresshoff, 1988). The only non-legumes known to form nodules with either *Rhizobium* or *Bradyrhizobium* belong to the genus *Parasponia* (Trinick and Galbraith, 1980) which are infected close behind the growing tip of the tap root. Bacteria colonize the root surface at this infection zone and begin to erode the mucilage layer of the primary cell wall of

epidermal cells within 24 hr of inoculation. Four days after inoculation, the overlying epidermal layer ruptures and a mass of dividing cells emerges. This region provides a site through which bacteria enter the root and colonize the root cortex. No root hair infection occurs in the invasion of *Parasponia* plants. Infection requires the initiation of cell division followed by the formation of infection threads from bacterial colonies within the root cortex and the induction of a lateral root below the site of cell division (Bender et al., 1987a,b). The final structure is a lateral root swollen on either side of the central vascular tissue by cortical cells filled with infection threads containing bacteria actively engaged in nitrogen fixation.

An important finding has been that some strains of rhizobia can nodulate both legumes and *Parasponia*, and we suggest that the widely differing nodulation processes are largely plant determined and require the correct trigger(s) from rhizobia. During these studies it was observed that not all rhizobia survive well within the cells of *Parasponia* plants or readily colonize their root surface. Some rhizobia are very rapidly 'rejected' by the plant's defences, while other strains multiply on the roots of *Parasponia* without any obvious plant reaction.

The genetic analysis of the nitrogen-fixing symbiosis between *Parasponia* and rhizobia has been a focus of the work on *Rhizobium* nodulation of non-legumes in Canberra since 1980 (Bender et al., 1987a,b; Plazinski et al., 1985; McIver et al., 1989). These studies of the different *Rhizobium* and *Bradyrhizobium* strains that can infect either legumes or *Parasponia* plants have shown that the conserved nodulation genes *nodABC*, needed for the curling of legume root hairs, were not essential for the initiation of infection, i.e. not required for the induction of cell division. However, these genes were required for *Parasponia* 'prenodule' development. In contrast, the regulatory *nodD* gene of *Rhizobium* strains was essential for the initiation of infection. In addition, successful infection required not only *nodD* but a host specific nodulation (*hsn*) region which is not needed for the nodulation of legumes. *Agrobacterium tumefaciens* carrying this *Parasponia*-specific region, as well as legume *nod* genes, was able to form nodules to an advanced stage of development on *Parasponia* plants. However, these nodules were non-nitrogen-fixing and had fibrillar material associated with the infection threads in the infected plant cells.

3. *Rhizobium* induced changes in the growth of root hairs on cereal roots

Earlier studies showed that specific recombinant *Rhizobium* strains containing the nodulation genes, *nodABCD*, could cause root hair curling and distortion (the Hac^+ / Had^+ phenotypes) on rice if they were carried on a vector which had an enhanced copy number (Plazinski et al., 1985). These findings showed that the expression of some of the *Rhizobium* symbiotic genes could induce changes in the growth of root hairs similar to that observed on legumes. The *nodABCD* genes are the minimum number of genes required for the formation of the extracellular metabolites which cause the Hac^+ phenotype on legumes. These nodulation factors are lipo-oligosaccharides (LOSs) (Lerouge et al., 1990) and if applied to the roots of white clover seedlings at a 10^{-10} M concentration the Hac^+ response occurred, while application at 10^{-8} M concentration induces both Hac^+ and cell division in the root cortex. Thus, the appropriate concentration of these LOS compounds can induce different plant cell responses. The implication of these findings on rice root hair curling was that *Rhizobium* metabolites will affect cells from both dicotyledonous and monocotyledonous plants.

Other studies support the view that as a result of the activity of the *Rhizobium* nodulation genes, the pattern of root hair growth on the monocotyledons oat, rice and maize can be altered (Terouchi and Syono; 1990a,b; Piana et al., 1988). Using microscopy studies, Terouchi and Syono (1990a,b) established that the presence of the Symbiotic (Sym) plasmid was necessary for *R. leguminosarum* bv. *trifolii* strains to attach to the root hairs of both oat and rice seedlings. In addition, only Nod^+ strains of *R. leguminosarum* bv. *trifolii* could reproducibly attach at a high frequency to the cell surface of wall-regenerating rice protoplasts made from rice mesophyll cells. The authors showed that *R. leguminosarum* bv. *trifolii* strains can extensively curl and distort the root hairs of oat seedlings without the addition of flavones, which had to be added to cultures of *R. meliloti* and *R. leguminosarum* bv. *viciae* strains before these latter *Rhizobium* strains could curl oat root hairs (Terouchi and Syono, 1990a). The possibility that oats excreted compounds which can induce *Rhizobium nod* genes of *R. leguminosarum* bv. *trifolii* strains was tested and confirmed, although the possibility that the root hair deformation response in oats might be more sensitive to *Rhizobium* extracellular products needs to be investigated. Piana et al. (1988) reported that a transconjugant strain, *Azospirillum brasilense* carrying the nodulation genes of *R. meliloti*, can induce root hair curling on maize and medicago seedlings.

4. Infection of Rice Seedling Roots

The results from three laboratories (in UK, China and Australia) indicate (1) that some *Rhizobium* strains can infect and induce nodule-like structures on the roots of rice seedlings and (2) that there does appear to be a large variation between rice varieties in their sensitivity to these *Rhizobium* strains, and (3) rhizobia because of their special properties (Table 1) appear to be the more desirable bacterial group to use in experiments when attempting to infect the roots of non-legumes with bacteria and induce a functional nitrogen-fixing 'nodule'.

Recently, it was found that the treatment of seedling roots with a mixture of cell wall-degrading enzymes, cellulase and pectolyase, could very rapidly remove the cell wall at the tip of root hairs of a wide range of crop plants, both legumes and non-legumes, including cereals (Cocking, 1985). The Nottingham group (Al-Mallah et al., 1987) extended these observations to show that root hairs of white clovers treated with cell wall-degrading enzymes (cellulase-pectolyase) removed a barrier to *Rhizobium*-host specificity and enabled the normally excluded *R. loti* bacteria, inoculated in the presence of polyethylene glycol (PEG) to infect plants at about 1% frequency and form nitrogen fixing nodules. This is an important observation not only because the enzyme treatment removes a barrier to infection but also to host specific regulation of nitrogen fixation as well. However, this enzyme-PEG treatment of *Lotus corniculatus* seedlings did not facilitate *R. leguminosarum* bv. *trifolii* to form nodules on *Lotus* seedlings, but only to induce deformation and some curling of the root hairs. Thus, the success of facilitating nodulation by the application of this protocol is dependent on the particular plant being tested.

Table 1. Advantages of *Rhizobium* for rice nodulation

- Both *Rhizobium* and *Bradyrhizobium* strains can colonize and infect the roots of legumes without eliciting plant defence systems.
- Rhizobia have evolved regulatory genes which respond to specific plant signals as part of the control of the expression of their invasion genes.
- Rhizobia can reside either intercellularly or intracellularly in legume roots without inducing host cell death responses.
- Both *Rhizobium* and *Bradyrhizobium* strains, which can fix nitrogen under *in vitro* conditions, are available.
- Different *Rhizobium* strains can often erode the mucigel slime layer and the surface epidermal cells of legume roots.
- *Rhizobium*-derived signals can distort plant cell differentiation and cell division, a prerequisite for nodule formation.

Subsequently, nodulation of non-legumes was also achieved at low frequencies by applying rhizobia in the presence of PEG and calcium chloride to enzyme-treated roots of both the monocotyledons, rice and wheat, and the dicotyledon, oilseed rape (Al-Mallah et al., 1989; Cocking et al., 1990). These nodule-like structures were induced on the roots of some varieties of rice and not others (Table 2). Taipei 177 was the most successfully infected rice variety, as nodule-like structures were formed after all enzyme-PEG treatments with different *Rhizobium* strains. However, *Rhizobium leguminosarum* bv. trifolii which induced up to 7% of the inoculated Taipei 177 plants to form 'nodules', failed to induce such structures on variety Taipei 309 (Table 2).

The nodular structures that formed were either spherical or elongated and their formation on rice seedlings roots depended on the use of the enzyme-PEG protocol. The internal organisation of these nodular structures was usually composed of irregularly-shaped plant cells, large air spaces and bacteria present in the intercellular spaces and within some of the outer layer cells of the 'nodule'. The plant cells associated with intracellular bacteria were either dead or degenerated. The bacteria were also often associated with fibrillar material (Al-Mallah et al., 1989). No nitrogen was fixed in these structures (Al-Mallah et al., 1989; Cocking et al., 1990).

A different approach to the nodulation of rice came from a group in China who reported that *Rhizobium* sp. from the legume *Sesbania cannabina* could

Table 2. Responses of rice varieties to *Rhizobium* inoculation

Characteristics of <i>Rhizobium</i> inoculant	IRNG rice nodulation experiments (% nodulation)			
	Nottingham (UK)		Beijing (PRC)	Canberra (Australia)
	Taipei 177	Taipei 309	Lianjiang Yuefu Jiangxi 80074	Calrose Echuca Lemont
Enzyme-PEG treated rice seedlings plus rhizobia				
<i>R. loti</i>	1.5	0.7		
<i>R. l.</i> bv. trifolii	7.3	0		
<i>R. loti</i> + <i>R. l.</i> bv. trifolii	1.7	2.5		
<i>Sesbania cannabina</i>				
Rice rhizobia strain Rr2			54.0-66.7	
Transconjugant <i>Rhizobium</i> strain ANU536				0.1-0.25

nodulate seedlings of some varieties of rice at a frequency of up to 67% (Jing et al., 1990; Li Guang-Shan et al., 1991). These workers used bacteria originally from *S. cannabina* to inoculate a hybrid rice variety DS. Small milky white nodules appeared within 15 days and these structures gradually became brown on their surface and grew to 2–3 mm in diameter by 35–40 days after inoculation. Bacteria were isolated from these rice 'nodules', purified and re-tested on the hybrid rice DS and three local Chinese varieties, Lianjiang, Yuefu, and Jianxi 80074, as well as *S. cannabina* (Table 2). These nodule-like structures were spherical and when sectioned were similar in construction to soybean and *S. cannabina* nodules. Within the 'nodules' there were structures interpreted to be infection threads, plant cells filled with bacteria encapsulated in membranes, and the bacteria had presumptive polyhydroxybutyrate (PHB) granules present. The general appearance was like that of an ineffective indeterminate nodule. In the earlier report from this group, nitrogen fixation was apparently observed by measuring acetylene reduction, however, adequate controls were not included. The important implications of these studies require extensive repetition in other laboratories before they should be widely accepted.

5. Construction of a Rice-Nodulating *Rhizobium* Strain

The genetic analysis of the different *Rhizobium* and *Bradyrhizobium* strains that infect *Parasponia*, along with the microscopic investigation of the *Parasponia* symbiosis, has enabled the construction of a *Rhizobium* strain which can nodulate rice without added phytohormones. This bacterial construction had the properties that *Rhizobium* strains required for the successful invasion of *Parasponia*: (1) an ability to induce erosion of the root surface layer of cells; (2) an ability to induce cell division in the sub-epidermal layer of root cells; and (3) contained an allelic form of the regulatory gene (*nodD*) which can interact with signal compounds produced by rice roots and cause induction of the normally unexpressed *Rhizobium* nodulation genes.

The basic *Rhizobium* strain *R. leguminosarum* bv. *trifolii* strain ANU 843, used in this construction, does not induce the defence responses of *Parasponia*, it erodes the root surface epidermal cells and induces root cortical cell division. Into a derivative of this strain (ANU 851, a *nodD* mutant) was inserted a *nodD* which was shown to respond to signals from the root exudates from rice seedlings and cause root hair distortions. This *nodD* gene was cloned into a high copy number vector to supply multiple copies of the regulatory gene and help ensure optimal *nod* gene regulation. This genetically engineered strain ANU 536 occasionally formed nodules on rice seedlings of variety Calrose

without any other additions at a low frequency of 0.25% (Fig. 1a and Table 2) (Rolfe and Bender, 1990). The nodule-like structures were small, white, and contained bacteria. Sections of one of the best structures had vascular bundles associated with the root outgrowth (Fig. 2a). One of these structures examined in some detail had an internal structure resembling that found within legumes and contained membrane encapsulated bacteria which often appeared to have inclusions resembling refractile granules of PHB (Fig. 2b). Furthermore, the infected plant cells did not appear stressed by the presence of the packaged bacteria.

So far we have been able to initiate nodule-like structures on three rice varieties readily available in Australia, short grain varieties, Calrose (Fig. 1a) and Echuca (Fig. 1c-e), and long grained variety, Lemont (Fig. 1b and Fig. 3). The seedling nodulation frequencies on these varieties were low, but similar in each case (between 0.10% to 0.25%) with small creamy white structures being formed (Table 2). Where bacteria were observed within these induced structures they were usually found between cells or in dead and dying plant cells. These bacteria also appeared to contain refractile PHB granules.

6. Future Developments

As an approach towards the development of *Rhizobium* inoculant for rice, an International Rice Nodulation Group was established by the Rockefeller Foundation to (1) select the best *Rhizobium*-rice cultivar combination that gives the highest reproducible frequency of nodulation; (2) use a detailed light and electron microscopy study of the infection of the roots of the rice seedlings to help design a more invasive inoculum strain; (3) to investigate the *Rhizobium* genetics and possible gene amplification processes that may be involved in the "adaptation" of rhizobia and their interaction with rice plants, and (4) use the above findings to modify this plant-microbe association into one that stimulates the growth of rice plants in the field. This stimulation could be due to bacterial-derived phytohormone effects, as well as to a contribution from fixed nitrogen.

The work is labour intensive and progress is slow, with a lot of variation in the frequency of formation of nodule-like structures. Identification of the various physiological factors that influence the induction of these root outgrowths is needed, although the growth status of the inoculated plants on nitrogen-free media and the pH of the growth media appear to be important for success.

One recent finding that might prove to be very important in strain development is the nodulation of oilseed rape in the absence of enzyme-PEG treatment when roots were inoculated with *Rhizobium* strain RP501 (Cocking et al., 1990)

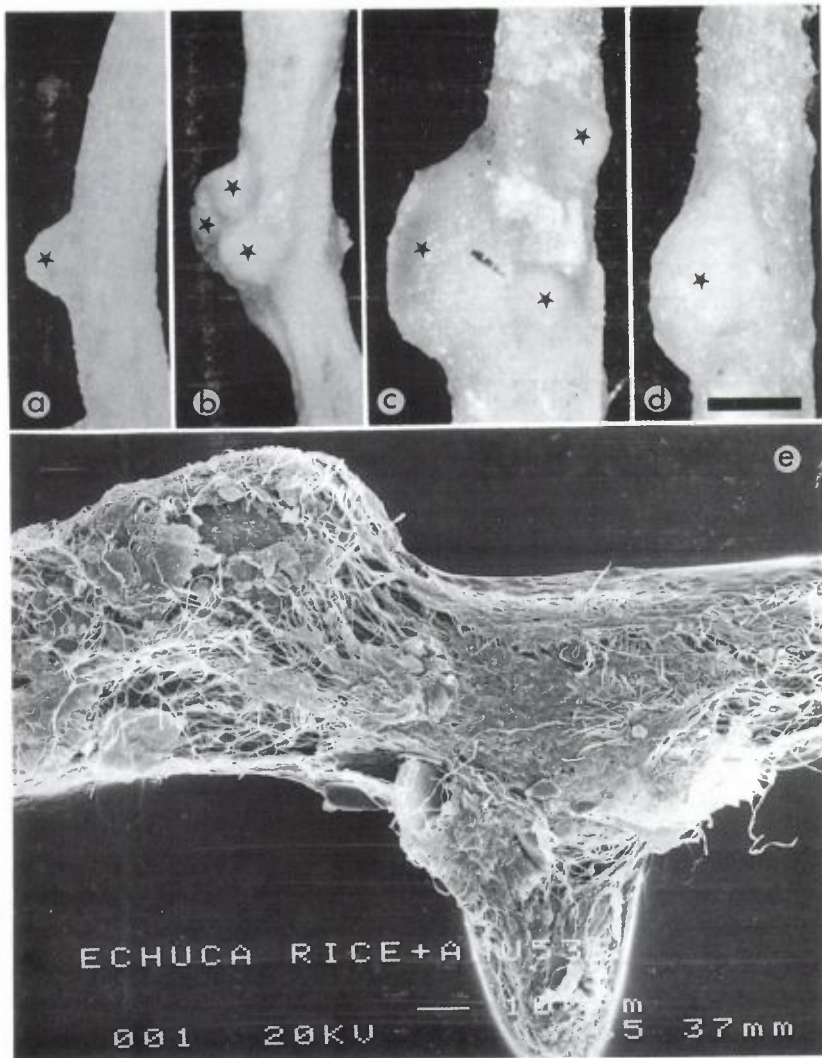


Figure 1. Nodule-like growths (stars in a-d) on three varieties of rice infected by the *Rhizobium* construct ANU536. (a) Short grain variety Calrose; (b) long grain variety Lemont; (c-e) short grain variety Echuca. In Figure e the nodule-like growth in the upper part of this scanning electron micrograph can be compared to a lateral root outgrowth in the lower part. Bar in Figure d represents 300 μm for Figures a-d. Bar in Figure e is 100 μm .

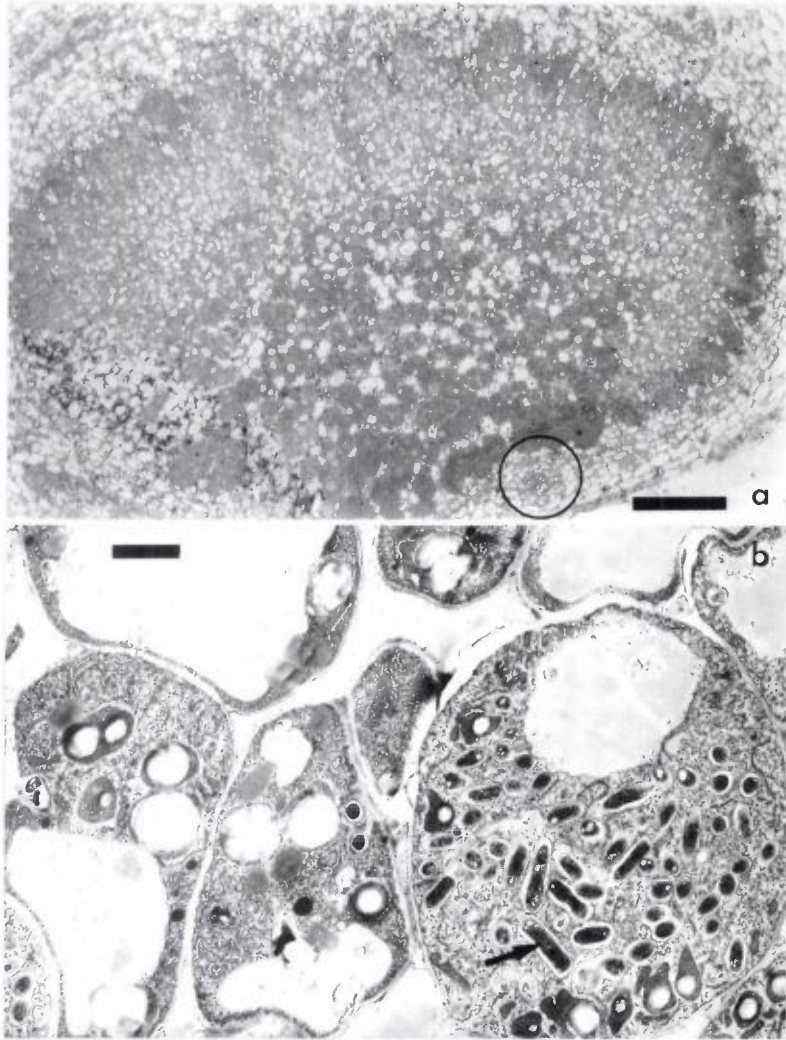


Figure 2. Sections of the best example of a nodule from the short grain variety Calrose infected by ANU536. The 'nodule' shows considerable internal structure, including vascular bundles (circled in a); bacteria (arrow in b) were found inside living cells, surrounded by a membrane. No infection threads were found. Bar in (a) = 30 μm ; Bar in (b) = 1 μm .

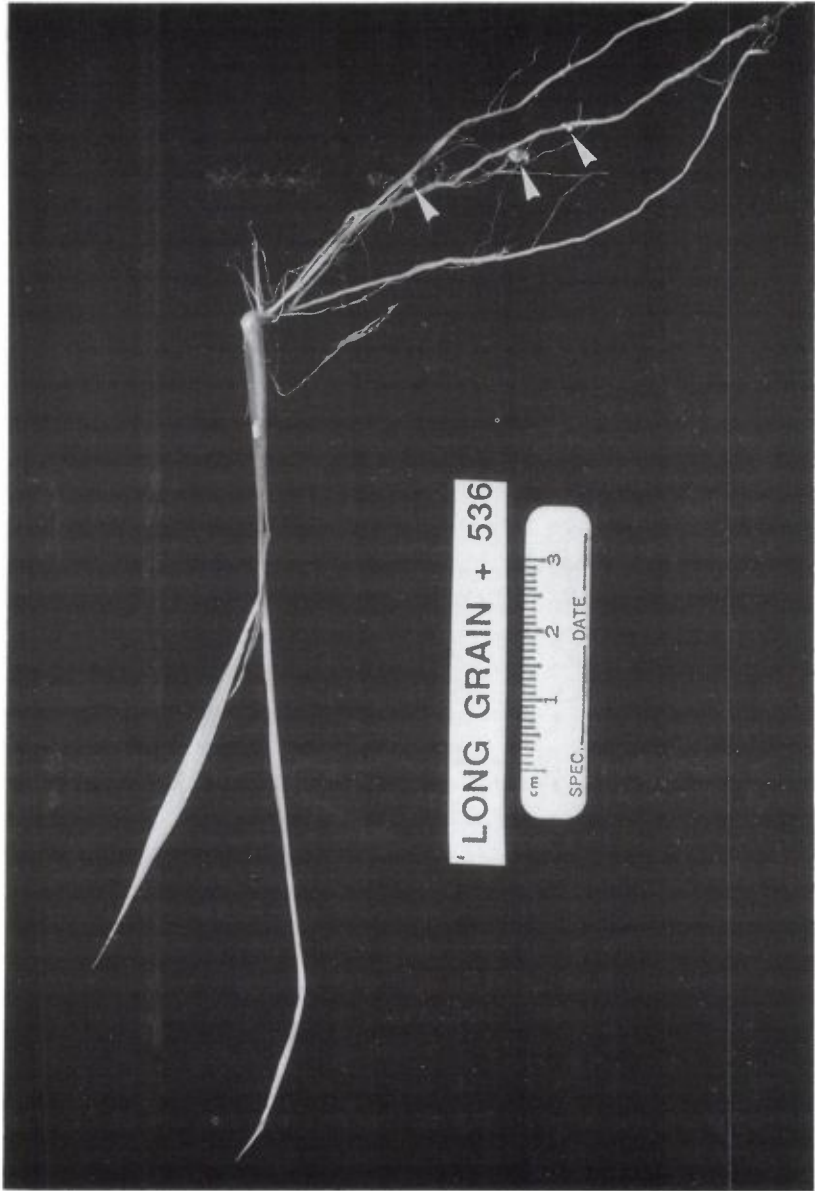


Figure 3. Nodule-like growths (arrowheads) on the long grain variety Lemont after infection by ANU536.

or *Bradyrhizobium* strain CP283 (Cocking, personal communication). Further analysis of the evolved invasion of the non-legume plant *Parasponia* might still be the quickest way to extend the host range to other non-legumes.

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REFERENCES

- Al-Mallah, M.K., Davey, M.R., and Cocking, E.C. 1987. Enzymatic treatment of clover root hairs removes a barrier to *Rhizobium*-host specificity. *Biotech.* **5**: 1319-1321.
- Al-Mallah, M.K., Davey, M.R., and Cocking, E.C. 1989. Formation of nodular structures on rice seedlings by rhizobia. *J. Exp. Bot.* **40**: 473-478.
- Al-Mallah, M.K., Davey, M.R., and Cocking, E.C. 1990. Nodulation of oilseed rape (*Brassica napus*) by rhizobia. *J. Exp. Bot.* **41**: 1567-1572.
- Bender, G.L. and Rolfe, B.G. 1985. A rapid plant assay for the *Parasponia-Rhizobium* symbiosis. *Plant Sci.* **38**: 135-140.
- Bender, G.L., Nayudu, M., Goydych, W., and Rolfe, B.G. 1987a. Early infection events in the nodulation of the non-legume *Parasponia andersonii* by *Bradyrhizobium*. *Plant Sci.* **51**: 285-293.
- Bender, G.L., Goydych, W., Rolfe, B.G., and Nayudu, M. 1987b. The role of *Rhizobium* conserved and host specific nodulation genes in the infection of the non-legume *Parasponia andersonii*. *Mol. Gen. Genet.* **210**: 299-306.
- Cocking, E.C. 1985. Protoplasts from root hairs of crop plants. *Biotech.* **3**: 1104-1106.
- Cocking, E.C., Al-Mallah, M.K., Benson, E., and Davey, M.R. 1990. Nodulation of non-legumes by rhizobia. In: *Nitrogen Fixation: Achievements and Objectives*. P.M. Gresshoff, Roth, L.E., Stacey, G., and Newton, W.E., eds. Chapman and Hall, London, pp. 812-823.
- Jing, Y., Li, G.H., Jing, G., Shan, X., Zhang, B., Guan, C., and Li, J. 1990. Rice root nodules with acetylene reduction activity. In: *Nitrogen Fixation: Achievements and Objectives*. P.M. Gresshoff, Roth, L.E., Stacey, G., and Newton, W.E., eds. Chapman and Hall, London, p. 829.
- Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C., and Denarie, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* **344**: 781-784.

- Li, G., Jing, Y., Shan, X., Wang, H., and Guan, C. 1991. Identification of rice nodules that contain *Rhizobium* bacteria. *Chinese J. Bot.* **3**: 8-17.
- McIver, J., Djordjevic, M.A., Weinman, J.J., Bender, G.L., and Rolfe, B.G. 1989. Extension of host range of *Rhizobium leguminosarum* bv. *trifolii* caused by point mutations in *nodD* that result in alterations in regulatory function and recognition of inducer molecules. *Molecular Plant-Microbe Interactions* **2**: 97-106.
- Piana, L., Delledone, M., Antonelli, M.N., and Fogher, C. 1988. Root hair deformation induced on maize and medicago by an *Azospirillum* transconjugant containing a *Rhizobium meliloti* nodulation region. In: *Azospirillum IV. Genetics, Physiology, Ecology*. W. Klingmüller, ed. Springer-Verlag, Berlin, pp. 83-91.
- Plazinski, J., Innes, R.W., and Rolfe, B.G. 1985. Expression of *Rhizobium trifolii* early nodulation genes on maize and rice plants. *J. Bact.* **163**: 812-815.
- Rolfe, B.G. and Gresshoff, P.M. 1988. Genetic analysis of legume nodule initiation. *Ann. Rev. Plant Physiol.* **39**: 297-319.
- Rolfe, B.G. and Bender, G.L. 1990. Evolving a *Rhizobium* for non-legume nodulation. In: *Nitrogen Fixation: Achievements and Objectives*. P.M. Gresshoff, Roth, L.E., Stacey, G., and Newton, W.E., eds. Chapman and Hall, London, pp. 779-780.
- Terouchi, N. and Syono, K. 1990a. Hair curling induced in heterologous legumes and monocots by flavonoid-treated rhizobia. *Plant Cell Physiol.* **31**: 113-118.
- Terouchi, N. and Syono, K. 1990b. *Rhizobium* attachment and curling in asparagus, rice and oat plants. *Plant Cell Physiol.* **31**: 119-127.
- Trinick, M.J. and Galbraith, J. 1980. The *Rhizobium* requirements of the non-legume *Parasponia* in relation to the cross-inoculation group concept of legumes. *New Phytol.* **86**: 17-26.