

## Altering the Specificity Control of the Interaction Between Rhizobia and Plants

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### Abstract

The finding that brief cell wall degrading enzyme treatment of the root hairs of legumes altered the specificity control of their interaction with rhizobia stimulated interest in whether the root epidermis was a major barrier to the initiation of nodule formation on non-legume crops. Studies using enzyme treated non-legume crops have indicated that nodule-like structures can be initiated by rhizobia on their roots. Similar nodule-like structures can also be formed on the roots of non-legumes by genetically engineered rhizobia. Recently, evidence has been obtained that naturally occurring *Parasponia* and *Aeschynomene* rhizobia can invade emerging lateral roots of rice, wheat and maize and initiate nodule formation, without the need for any enzyme treatment.

Keywords: *Aeschynomene*, nodules, non-legumes, *Parasponia*, rhizobia, specificity

## 1. Introduction

The establishment of an International Rice Nodulation Group by the Rockefeller Foundation, involving co-ordinated research in Australia, China, England and Mexico, has renewed interest in devising ways to nodulate non-legume crops for symbiotic nitrogen fixation (Cocking and Davey, 1991). As surveyed at this International Symbiosis Congress, these and other research groups are now attempting to extend the *Rhizobium*-legume symbiosis to non-legumes by utilising various bacterial strains and environmental conditions to induce nodule-like structures on roots (Ridge et al., 1991).

The symbiotic nodular fixation of atmospheric nitrogen by legumes was discovered more than a century ago, and early this century some preliminary trials were performed attempting the nodulation of non-leguminous plants with legume rhizobia. However, conclusive results were not obtained (Cocking and Davey, 1991). Plants of the genus *Parasponia* are the only non-legumes known to be nodulated by a diazotrophic *Rhizobium*, and existence of nodulated *Parasponia* illustrates the possibility of extending rhizobial symbioses beyond the Leguminosae (Postgate, 1987). The need for more effort to extend the range of symbioses between rhizobia and crop plants has been highlighted (Borlaug, 1970), but, until recently, there seemed little likelihood of extending symbiotic associations between rhizobia and legumes to non-legume crops.

Brief treatment of the root system of legumes, such as clover, with cell wall degrading enzymes has been shown to remove a barrier to *Rhizobium* specificity (Al-Mallah et al., 1987), thereby altering the specificity control of rhizobial interaction. Nodule-like structures have also been produced on rice, wheat and oilseed rape by naturally occurring rhizobia, following enzyme treatment of the root systems (Cocking et al., 1990). Genetically engineered strains of rhizobia have been used to induce nodule-like structures on the roots of rice (Jing et al., 1990; Li et al., 1991; Rolfe and Bender, 1990).

We have also observed that naturally occurring wild type *Rhizobium parasponium* RP501, which produces nodules of lateral root origin on *Parasponia*, can produce nodule-like structures on the roots of oilseed rape in the absence of any enzyme treatment (Cocking et al., 1990). More recently, we have observed a somewhat similar response on oilseed rape with *Bradyrhizobium* strain CP283, which also produces nodules of lateral root origin on *Parasponia*. Most of these nodule-like structures on oilseed rape resembled shortened, swollen lateral roots. This led us to investigate whether rhizobia, naturally nodulating *Parasponia*, would also nodulate cereals such as rice, under our test conditions, which involves inoculation of surface sterilised seeds with rhizobia and their germination on a medium lacking fixed nitrogen. Because it had been

established in tropical woody legume *Aeschynomene* spp. that nodulation on stems occurs at predetermined sites, identified as adventitious root primordia (Alazard, 1985), *Aeschynomene* rhizobia were also utilised in our nodulation studies.

## 2. Materials and Methods

### *Growth of plant material and inoculation with rhizobia*

Seeds of rice (IR36) were supplied by the USDA and seeds of wheat (variety Wembley) and maize John Innes hybrid by PBI Cambridge. Seeds were surface sterilised by immersion in 70% ethanol for 30 sec, then in 0.1% SDS and 0.1% HgCl<sub>2</sub> for 5 min followed by immersion in 30% Domestos bleach (Lever Bros, London) for 40 min and rinsed 4 times with sterile distilled water. Sterilised seeds were placed onto plates of RH medium (Al-Mallah et al., 1987) and incubated in the dark at 28°C for 4 days. Germinated seeds were then inoculated with a 3-day old rhizobial liquid culture and left to soak for 30 min. Inoculated seedlings were then aseptically placed onto 50 ml autoclaved RH medium solidified with 0.25% w/v Phytigel (Sigma) in 6 oz Powder Round glass jars (Beatson Clarke & Co., Ltd., Rotherham, Yorkshire).

### *Rhizobium cultures*

*Rhizobium* ORS 310 naturally nodulating *Aeschynomene* spp. was supplied by Dr. D. Alazard, Senegal, and *Bradyrhizobium* CP283 naturally nodulating *Parasponia* spp. by Prof. J. Sprent, Dundee. ORS 310 and CP283 rhizobia were maintained on YEM medium, and bacterial suspensions were prepared as previously described (Al-Mallah et al., 1989).

### *Light and electron microscopy*

Material for electron microscopy was prepared as previously described (Al-Mallah et al., 1987). Sections (2.0 µm) for light microscopy were stained with 0.5% w/v toluidine blue in 0.1% sodium tetraborate (3 min, 60°C).

## 3. Results and Discussion

### *Studies using rice*

Sterilized seeds of the widely cultivated rice variety IR36 were treated with *Bradyrhizobium* strain CP283 and germinated on a medium lacking fixed nitrogen. Lateral roots were observed which were shorter and thicker than lateral

roots formed on rice seedlings in the absence of rhizobia. These short, thickened lateral roots were fixed and embedded for examination using both light and electron microscopy. Cells of the lateral root cortex appeared deep blue when stained with toluidine blue, and electron microscopy, with its greater resolution, showed rhizobia within cells of the cortex (Fig. 1, A and B) and between cells of the cortex (Fig. 1, C and D). The root system of IR36 seedlings was poorly developed in the medium employed which lacked fixed nitrogen (Al-Mallah et al., 1987). This made it difficult to assess quantitatively the frequency of this interaction of rhizobia with emerging lateral roots, when using IR36.

#### *Studies using wheat*

The nodulation of the stems of *Aeschynomene* depends on the accessibility of root primordia on the stems to rhizobial invasion (Alazard, 1985). This led us to investigate whether *Aeschynomene* rhizobia (ORS 310) would initiate nodulation of wheat by invasion of lateral root primordia. Sterilized seeds of wheat variety Wembley were treated with ORS 310 and germinated on a medium lacking fixed nitrogen. During a period of up to 4 weeks, under our test conditions, the root system developed extensively, and lateral roots were observed that were shorter and thicker than the lateral roots formed on wheat seedlings grown on the same medium, but in the absence of rhizobia. Following fixation and embedding, toluidine blue stained sections of these thickened lateral roots showed large numbers of rhizobia between cells of the cortex overlaying emerging lateral root primordia, and also within some of the cells of this cortex. Since the frequency of this response could be more readily quantified in wheat, due to the extensive rooting system that develops under the conditions employed, we are currently investigating the detailed developmental stages in the interaction of emerging lateral root primordia with *Aeschynomene* rhizobia. A developmental study such as this is required to determine whether the mode of intercellular invasion in wheat is comparable to that in the legumes, *Arachis* (Chandler, 1978), *Stylosanthes* (Date and Roughley, 1982) and *Aeschynomene* (Alazard and Duhoux, 1987), where emerging lateral roots cause a disruption of the epidermis, allowing rhizobial entry via intercellular spaces. Similar studies are also being undertaken utilising *Parasponia* rhizobia.

#### *Studies using maize*

Sterilized seeds of the maize variety John Innes hybrid were treated with *Aeschynomene* rhizobia (ORS 310) and germinated on a medium lacking

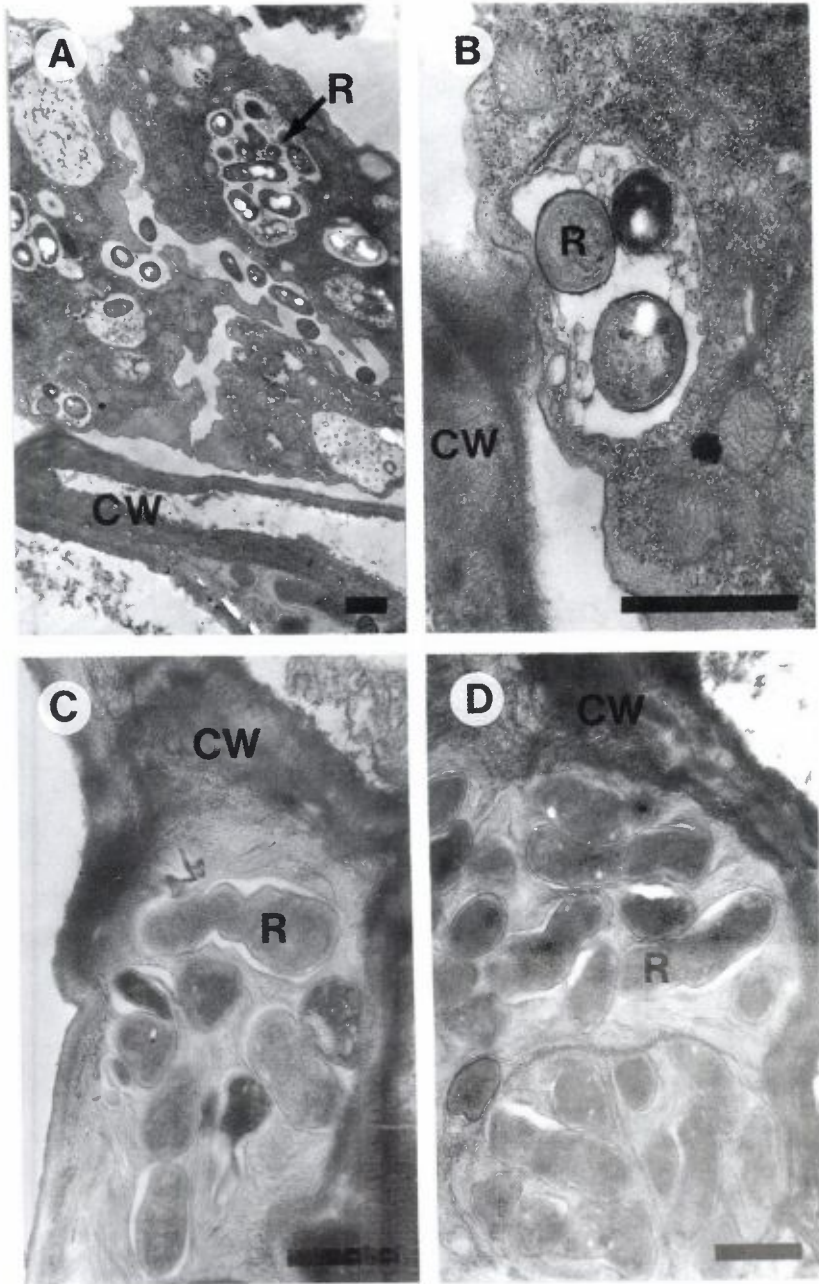


Figure 1. Transmission electron micrographs of rice (variety IR36) lateral roots inoculated with *Parasponia Rhizobium* strain CP 283.

A, B.

Rhizobia within membrane-bounded vesicles in the cytoplasm of a lateral root cortical cell.

C, D.

Rhizobia, surrounded by microfibrillar material, between the walls of lateral root cortical cells.

cw = cell wall, R = Rhizobia, bar = 1  $\mu$ m.

fixed nitrogen. The root system developed extensively, and a developmental study is being undertaken on the interaction of emerging lateral roots with *Aeschynomene* rhizobia under our test conditions by sampling lateral roots at weekly intervals. After 2 weeks, small, thickened lateral roots were selected for fixation and embedding. Examination of toluidine blue stained sections by light microscopy showed that rhizobia invaded the base of the emerging lateral root primordia, and penetrated between cells at the base of the primordia. At 4 weeks, thickened lateral roots, which were fixed and embedded, were examined using both light and electron microscopy. It was observed that rhizobia had penetrated extensively between the cells of the cortex of the lateral root towards the apex. Currently, we are investigating whether host cells of the cortex of these lateral roots can be invaded from the intercellular rhizobia by infection thread formation, as observed in the early development of *Rhizobium*-induced root nodules of lateral root origin in *Parasponia rigida* (Lancelle and Torrey, 1985). As for wheat, similar studies are also being undertaken utilising *Parasponia* rhizobia.

#### *Future studies using cereals*

It is now important to establish whether there is a general pattern of interaction of naturally occurring rhizobia such as those that nodulate *Parasponia* and *Aeschynomene*, and other genetically engineered rhizobia, with the emerging lateral roots of cereals, and to determine the detailed developmental pathway of nodule formation. As stressed by Ridge et al. (1991) at this International Congress, it is also essential to obtain a better understanding of the complex cytological and biochemical events that underlie these interactions, and the development of any nitrogen-fixing symbioses. In addition, it will be important to analyse any genomic rearrangements in rhizobia utilising the approaches already developed in legumes (Romero et al., 1991).

Nitrogen fixation is apparently the result of and not a prerequisite for nodule development. It is therefore to be expected that effective nodulation of cereals arising from the interaction of these tropical rhizobia with emerging lateral roots will only possibly occur when full nodule development has taken place. Currently, we are using acetylene reduction and  $N^{15}$  isotope dilution assays to assay for any nitrogen fixing capability at various stages in nodule development. As yet, no positive results have been obtained using seedlings growing in jars interacting with rhizobia; currently, seedlings are being grown under better physiological conditions and assays for nitrogen fixation are continuing.

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