Interactions of rice seedlings with bacteria isolated from rice roots

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Abstract. The interactions between two groups of rice endophytic bacterial strains and several rice cultivars were investigated. Various strains of \textit{Rhizobium leguminosarum} \textit{bv. trifolii}, originally isolated from rice plants grown in Egypt, comprise one group. The second group of bacterial strains was isolated from rice cultivars grown in the Philippines. Inoculation experiments with rice seedlings showed that specific isolates of these rice-associating bacteria could either promote, inhibit, or have no influence on rice plant growth. Furthermore, these growth effects were greatly influenced by the environmental growth conditions used. Studies to examine root colonisation patterns, using \textit{Rhizobium} strains into which a plasmid expressing the green fluorescent protein has been placed, showed that the bacteria preferentially colonise rice seedling surfaces mainly in clumps. This occurs along grooves on the rice root surface, or at the emerging lateral root zones and at the root tips. However, rhizobia could also colonise intercellularly in lateral roots formed on the main roots near the culm region of the seedling. Under the growth conditions used, this occurred most frequently with strain R4 which multiplied and migrated to form long lines of individual bacterial cells along the inside of growing lateral roots. A bioassay to measure bacterial multiplication in rice leaves showed that the rice-associating strains can multiply and survive at different rates within these tissues. They were not, however, detected migrating into other parts of the leaf from the original site of pressure-infiltration, indicating that the bacterial ability to migrate within the lateral roots is not matched by a similar capacity in rice leaves. We suggest that some of these rice-associating bacteria possess important genes that enhance their ability to intimately colonise niches on and within rice tissues, and promote rice plant growth.

Keywords: \textit{Rhizobium}; root colonisation; green fluorescent protein, rice, endophytes.

Introduction

Agriculturalists have searched for various soil bacteria that might make reliable contributions to non-legume plant growth (Bennett and Ladha 1992; Ladha \textit{et al}. 1997; Rolfe \textit{et al}. 1997, 1998a). Organisms that were plant growth-promoting rhizobacteria (PGPR) were used as inocula to enhance crop growth (Kloepper \textit{et al}. 1992). Some of the factors thought responsible for PGPR effects are the bacterially-produced phytohormones (Costacurta and Vanderleyden 1995). The most widely used group of bacterial inocula for non-legumes have been the nitrogen-fixing \textit{Azospirillum}, which have been applied to different crops with some success at increasing yields, possibly via PGPR effects (Okon and Labandera-Gonzalez 1994; Okon \textit{et al}. 1995). Recently, the isolation from rice seedlings of two groups of diazotrophic endophytic bacteria (micro-organisms found inside roots as internal root colonists, Dickinson and Lucas 1982; Kloepper \textit{et al}. 1992) has been reported (Barraquio \textit{et al}. 1997; Ladha \textit{et al}. 1997; Yanni \textit{et al}. 1997; Dazzo \textit{et al}. 1998).

In the Philippines, rice plants have been identified which harbour a wide spectrum of endophytes in their tissues, and exhibit to some degree a varietal discrimination in forming these associations (Barraquio \textit{et al}. 1997; Stoltzfus \textit{et al}. 1997). The endophytes were diverse types of nitrogen-fixing and non-nitrogen-fixing bacteria, and were found mainly in the root and culm regions. Some were also found in the seeds of different cultivated rice species (Barraquio \textit{et al}. 1997; Stoltzfus \textit{et al}. 1997).

In Egypt, rice and berseem clover have been rotated annually for more than 700 years, thus ensuring a population of rhizobia in the soils used for rice growth. Isolates of \textit{Rhizobium leguminosarum} \textit{bv. trifolii}, which are normally soil saprophytes or legume symbionts, have been found within the roots of rice plants that had been grown in these fields (Yanni \textit{et al}. 1997). These strains were found to naturally colonise the rice roots and could promote rice growth under laboratory conditions. The extent of these growth responses was influenced by the rice cultivar, the inoculant strain, the plant growth medium, and the growth parameter.
measured. Furthermore, two field experiments have shown that inoculation of rice seedlings with two of these endophytic *Rhizobium* strains can significantly increase grain yield and agronomic N-use efficiency of the rice plants (Yanni et al. 1997; Dazzo et al. 1998). The molecular and genetic characterisation of these endophytic strains has only just begun (Yanni et al. 1997; Rolfe et al. 1998b).

The present study was initiated to investigate the biological properties of the rice-associated bacterial strains isolated in Egypt and the Philippines. We have adapted and developed a standard set of growth assay techniques, and used these to investigate the consequences of the microbial interactions with several rice cultivars. This standardisation is necessary so that variations due to mutation or complementation can be easily assessed, and enables the establishment of a genetic basis for the microbe-rice plant interactions. We present initial studies of the growth effects of bacterial inoculation, location and nature of the bacterial colonisation with rice seedlings, and the ability of these endophytes to grow within rice plant tissues.

**Materials and methods**

### Bacterial strains

All bacterial strains used in this study are listed in Table 1. Plasmid pTB93Fa was used (Gage et al. 1996). Rice-associating *Rhizobium leguminosarum* bv. *trifolii* strains were isolated from either the surface-sterilised root tissues (E strains) or the root rhizosphere (R strains) of rice plants grown at Sakha Kafr El Sheikh in the middle Nile delta region of Egypt (Yanni et al. 1997). The archetypal *R. l.* bv. *trifolii* strain ANU843 (Rolfe et al. 1980), a non-rice-associated bacterium, was used as a control. The unidentified endophytic bacterial strains were isolated by IRRI scientists in the Philippines from surface-sterilised rice root tissues (Barraquio et al. 1997).

### Media

**Bacterial growth media**

Bacterial strains were grown on Bergersen’s modified medium (BMM) at 29°C for 3 days before inoculation of plants (Rolfe et al. 1980). Bacteria for inoculation were suspended in sterile water.

### Plant growth media

Nitrogen-free Modified Fåhraeus medium (NFM) has been described previously (Rolfe et al. 1980; Rolfe and McIver 1996). Where specified, 10 mM KN0₃ was added to this medium. Hoagland #2 medium (Sigma Chemical Co.) contains 15 mM nitrogen as 6 mM KN0₃, 4 mM Ca(NO₃)₂ and 1 mM NH₄H₂PO₄. Agar at 1.2% was added to media to make slopes in cylindrical specimen jars and used in 30-day growth-assessment experiments.

### Plant growth studies

Three rice cultivars, Calrose, Pelde and IR-28 were used to examine the effect of several Egyptian rice-associated *Rhizobium leguminosarum* bv. *trifolii* isolates, and a number of unidentified endophytic strains from IRRI on the growth of rice. Seeds were obtained from Dr L. Lewin, Agriculture NSW, and were dehusked, rinsed in 95% ethanol for 3 min and then surface sterilised in 1 part sodium hypochlorite (50 g L⁻¹ of available chlorine as sodium hypochlorite) to 3 parts sterile water for 30 min. The seeds were then washed four times with sterile water before being transferred to petri dishes containing BMM agar. About 20 seeds were put on each plate, and melted sterile 0.8% water agar was pipetted over the seeds to hold them in place. This assisted germination of the seeds. They were then incubated in the dark at 29°C for three days to allow germination.

The system for testing rice plant growth which gave the most reliable and reproducible results for up to 30 days was found to be the use of agar slopes in 10.5-cm high (100 mL) specimen jars (Bunzl). These jars contained 83 mL of 1.2% solid agar medium as a slope, and one seedling per jar. The jars containing plants were incubated in a growth chamber with a photon flux density of 650 µmol m⁻² s⁻¹ using a 12-hour 30°C day and a 12-hour 20°C night cycle with 70% relative humidity. Initially the jars were covered with plastic bags with one corner removed, to help protect the immature seedlings from desiccation and to prevent contamination. After 7 days, the bags were removed, and liquid medium of the same composition as in the agar slope was added to cover the roots and stem base. This was replenished as levels dropped. The tops of the jars, except for the protruding shoot of the rice seedling, were then sealed with Nescofilm (Bando Chemical Ind. Ltd, Kobe, Japan) to limit external contamination of root system. The plants were watered with the liquid medium as the level of liquid medium dropped to the stem base.

This method gave more consistent results than agar in test tubes or on large petri dishes, and was simpler than using vermiculite in Magenta jars. The resulting plants were less stressed, developed rapidly, and gave good plant growth over a 30-day period.

Six replicates were used in each treatment and experiments were repeated at least once. Rice plants were harvested 30 days after inoculation and the roots were carefully rinsed with water to remove any growth sub-

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### Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td>From ANU:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANU843</td>
<td>Wild type <em>R. l. bv. trifolii</em> Nod⁺ Fix⁺ on clovers</td>
<td>Rolfe et al. (1980)</td>
</tr>
<tr>
<td>ANU843(gfp)</td>
<td>ANU843 containing pTB93Fa</td>
<td>This study</td>
</tr>
<tr>
<td>Originally from Egypt:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4, E11, E12</td>
<td><em>R. l. bv. trifolii</em> Nod⁺ Fix⁺ on clovers from sterilised rice tissues</td>
<td>Yanni et al. (1997)</td>
</tr>
<tr>
<td>E4(gfp)</td>
<td>Strain E4 containing pTB93Fa</td>
<td>This study</td>
</tr>
<tr>
<td>E11(gfp)</td>
<td>Strain E11 containing pTB93Fa</td>
<td>This study</td>
</tr>
<tr>
<td>E12(gfp)</td>
<td>Strain E12 containing pTB93Fa</td>
<td>This study</td>
</tr>
<tr>
<td>R2, R4</td>
<td><em>R. l. bv. trifolii</em> Nod⁺ Fix⁺ on clovers from the rice root rhizosphere</td>
<td>Yanni et al. (1997)</td>
</tr>
<tr>
<td>R4(gfp)</td>
<td>Strain R4 containing pTB93Fa</td>
<td></td>
</tr>
<tr>
<td>Originally from IRRI:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R38-0, R38-T</td>
<td>Presumptive endophyte isolated from <em>Oryza alata</em></td>
<td>Barraquio et al. (1997)</td>
</tr>
<tr>
<td>R53, R58</td>
<td>Presumptive endophyte isolated from <em>Oryza eichingeri</em></td>
<td>Barraquio et al. (1997)</td>
</tr>
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</table>
To examine the ability of the bacteria to colonise internal plant tissues, and comparing their performance to that on roots. Bacterial cells were forced by pressure into the intercellular spaces of leaf sections of the youngest leaf, and bacterial viable counts were recorded at intervals over 15 days. These assays were based on the current bacterial plant pathology leaf assays, which demonstrate that bacterial cells can grow and multiply within leaf tissues only if they contain the hrp genes. Strains with mutations in this cluster of genes do not grow in plant tissues. Part of the function of the hrp genes appears to be the uptake of different nutrients. Two rice cultivars, Calrose and Pelde, were used, and the seeds were dehusked, sterilised and treated as described earlier for the growth studies. Germinated seedlings were planted in vermiculite in Magenta jars, two plants per jar, and watered with MF 15, a modified NFM containing MgSO4 at 2 mM with nitrogen added in the same form and concentration as in Hoagland #2. After 3 or 4 weeks, individual young leaves of rice plants were inoculated with a suspension of about 10^7 per mL bacteria. This was done by pressing the blunt end of a sterile polyethylene disposable transfer pipette (BioRad, CA, USA) to the upper (adaxial) side of the leaf and forcing the suspension into the leaf tissue. This process causes mild damage at the infiltration site, and aids bacterial entry, but has no long-term damage of the leaf as evidenced by its continued growth. Four overlapping applications were made to each leaf. A marking pen was used to identify the location of the region of the leaf used for this pressure-infiltration. The excess bacterial suspension was removed by blotting. This procedure delivered between 10^2 and 10^3 bacterial cells into the leaf tissue at the pressure-infiltration site, determined by assay 1 h after application.

To harvest leaf tissue, a 2-cm segment of the leaf with the infiltration site was excised and sterilised in sodium hypochlorite (1.2% w/v available chlo-
ride) for 10 min to kill surface organisms. Each individual leaf section was cut up with scissors and put into an Eppendorf tube containing 20 mg ground glass (particle size 0.2–0.3 mm diameter, Schott glass, Jena, Germany) and 50 mL of protoplast dilution buffer (PDB) (Gresshoff and Rolfe 1978). The plant tissue was then ground using a polypropylene pellet pestle (Kontes, NJ, USA) in a variable speed impact drill until the sample contained no obvious leaf pieces. To prevent overheating during the grinding procedure, the Eppendorf tube was placed into a beaker containing an ice slurry. Samples from the ground suspension were then diluted and plated out onto BMM plates. Three leaves were assayed separately for each strain at each sample time. The experiments were repeated at least three times for each strain. Harvesting was carried out 1 h after inoculation (Day 0) and then at 2, 6, 9 and 15 days after inoculation.

Results

Effect of the bacterial strains on rice growth

Cultivar IR-28 inoculated with strain R4 and grown in test tubes gave the greatest growth stimulation; this was significant at the \( P = 0.05 \) level (data not shown). The growth of all other rice–Rhizobium combinations was not significantly stimulated. Rice plants from each cultivar inoculated with strain E12 gave the poorest growth results. However, this procedure gives rise to rice plants that are stressed and developed poorly over the 30-day period. The average shoot dry weight of the uninoculated plants ranged from 70–180 mg. Hence, we trialed other methods for growing rice plants that would be less labour intensive and would optimise plant growth over the 30-day period.

The cylindrical specimen jar method was used for growth assessment in medium-term (30-day) experiments. This procedure used Hoagland’s growth medium, and routinely gave average shoot dry weights of the uninoculated plants in the range of 500–1100 mg, depending on the rice cultivar.

Growth of the rice cultivars Calrose, Pelde and IR-28 was examined by the cylindrical specimen jar method following inoculation with a variety of rice-associating bacterial isolates. The strains tested were Rhizobium strains R2, R4, and E4, and the IRRI isolates R38-0, R38-T, R53, and R58. Each of the three cultivars gave different growth responses to bacterial inoculation (see Fig. 1). On NFM the IRRI strain R38-T significantly enhances the growth of rice seedlings of cultivars Pelde and Calrose. The growth of cv. IR-28 seedlings was enhanced by inoculation with strain R38-T, but inhibited by strain R53 inoculation. However, these growth effects were not statistically significant.

When the same experiments were performed with 10 mM KNO\(_3\) added to the NFM growth medium, the Rhizobium strain R4 significantly stimulated the seedling growth of cv. Pelde and IR-28. Similarly, both the IRRI strains R38-0 and R38-T significantly stimulated the growth of cv. Pelde seedlings (Fig. 2). The IRRI strain R58 significantly stimulated the growth of cv. IR-28 seedlings. Under these conditions, most of the bacterial inoculations had little effect on the growth of cv. Calrose seedlings (Fig. 2). However, the Rhizobium strain E4 and the IRRI strain R53 significantly inhibited seedling growth and development in all three culti-

Fig. 1. Shoot dry mass of inoculated rice seedlings 30 d after inoculation of cultivars (A) Pelde, (B) Calrose and (C) IR-28, all grown on nitrogen-free F medium and inoculated separately with different bacterial strains. Control (CTR) was uninoculated rice plants (bars indicate least significant difference LSD, \( P = 0.05 \)).
Fig. 2. Shoot dry mass of inoculated rice seedlings 30 d after inoculation of cultivars (A) Pelde, (B) Calrose and (C) IR-28. The seedlings were inoculated separately with different bacterial strains and grown either on nitrogen-free F medium + 10 mM KNO₃, or on Hoagland’s medium. Control (CTR) was uninoculated rice plants and the experiments repeated at least once (bars indicate least significant difference LSD, $P = 0.05$).
On Hoagland's medium, all bacterial inoculum strains had minimal growth effects on the development of the various rice cultivars (Fig. 2). The use of this plant growth medium greatly reduced the inhibition of seedling growth caused by strains E4 and R53. The basis of the modulation of the growth inhibition by Hoagland's medium was investigated by changing the various media compositions in an attempt to define the crucial constituents involved. As 12 different media combinations were compared in this series of experiments, two plants were grown per specimen jar. As a consequence, growth of the individual seedlings by day 30 was reduced, but the original differences between F10 and Hoagland's media were still very apparent. Our studies indicate a complex interaction occurring between the levels of Ca, K+ and phosphate ions and nitrogen addition (Fig. 3). Rice seedlings grown in medium F10#6 combination were similar to that grown in Hoagland's medium. Seedlings grown in all other medium combinations were significantly poorer in growth.

We examined the roots in those cases where growth interactions were observed. Where plant growth enhancement took place, it was always accompanied by extensive lateral root and root hair formation (Figs 4A, B). Conversely, when rice seedling growth was severely inhibited, there was a marked reduction of the number of main and lateral roots, and in root hair formation along the main roots that did form (Fig. 4C). In the case of the IRRI strain R53, inhibition of rice seedling growth was accompanied by a major reduction in the total number of main roots formed. Lateral roots were very stunted and the formation of root hairs was greatly reduced and inhibited in their development (Fig. 4E). In contrast, the seedling growth inhibition induced by strain E4 was not accompanied with an obvious effect on the formation of these root structures (Fig. 4D).

Having demonstrated enhanced rice growth, cases of growth inhibition and environmental effects on the responses of rice seedlings, we made use of a number of techniques to further characterise the bacterial colonisation of the rice seedling roots.

Colonisation of rice roots

We followed the colonisation of rice roots by a number of *Rhizobium* strains tagged with gfp to enable bacterial colonisation to be followed with fluorescence microscopy. The strains used varied in their ability to influence rice seedling growth. Three different methods of incubating inoculated rice seedlings with tagged bacterial strains were investigated; seedlings grown on (a) agar plates, (b) in 20 mL of liquid medium in McCartney bottles, and (c) in 250 mL of liquid medium in Magenta jars. The results are shown in Figs 5–7.

**Method a: seedlings grown on agar plates**

*At day zero.* Within 2 h of inoculation the bacteria were seen moving near root hairs, or attached to the cell surfaces of the root hairs. Examination under the fluorescence microscope showed that strains R4(gfp) and ANU843(gfp) were observed to move more actively in the fluids around the rice seedling roots compared to the bacterial strains E11(gfp) or E12(gfp).

*At day 3.* Bacteria were associated with the rice root system (Fig. 5). Bacteria could be seen along the grooves of the surface epidermal cell files (root grooves) of the main roots and in microcolonies on the root surfaces (Fig. 5D), often at the junction of a lateral root (Fig. 5A). The rice seedling root hairs were not curled or distorted, as is usually observed with host legume root hairs.

*At day 7.* Strains R4(gfp) and ANU843(gfp) were located along the root grooves, with microcolonies on the root surfaces on the old and new main roots. Bacterial colonisation of the root surface was not extensive, and occurred in clumps along the root (Fig. 5D). In addition, on 60% of the originally inoculated plants these two strains were found to infect some of the cells of the roots (Fig. 5E). Bacteria were observed to move about inside these plant cells. These ‘infected’ plant cells were only observed on inoculated roots, and were located within an area corresponding to a site 2–3 cm below the culm region of the seedling at the time of inoculation. Strains R4(gfp) and ANU843(gfp) were also noted on the root tip (Figs 5B, C) and around the points of emerging lateral roots. However, the distribution of the strains along
the root surfaces was not the same. In the ten plants examined, these bacteria grew more extensively near the culm region and less toward the root tip.

Strains E11(gfp) and E12(gfp) predominantly occupied the culm region of the rice seedling and grew less extensively along the root. These strains colonised the root grooves along the root less extensively than strains R4(gfp) or ANU843(gfp), which formed patches of longer regions of bacterial growth along the root grooves. Strains E11(gfp) and E12(gfp) were also observed on the root tip and around the points of emerging lateral roots (Fig. 5). Overall, the rootlets formed after the bacterial inoculation with strains E11(gfp) and E12(gfp) had less bacteria present or, on some seedlings, even an absence of detectable bacteria.

Two weeks after inoculation, there were no changes to the patterns of root colonisation by the various bacteria that were seen seven days after inoculation.

Methods b and c: colonisation of rice seedlings grown in liquid media

Root hair attachment

Seedlings were grown in liquid medium in McCartney bottles. We compared strains R4(gfp) (a growth-promoting strain), E4(gfp) (a growth-inhibiting strain) and ANU-
Fig. 5. Root colonisation visualised under fluorescence microscopy with *Rhizobium* strains tagged with the green fluorescent protein (gfp). 3-d-old cultivar Pelde seedlings were inoculated separately with different *Rhizobium* strains E11(gfp), E12 (gfp), R4(gfp) and ANU843(gfp). The results shown here are for strain R4(gfp), but a similar type of response was found for each strain: (A) bacteria congregating at an emerging lateral root site; (B) at the root tip; and (C) the same root under bright field. At 7 days: (D) individual cells and microcolonies of *Rhizobium* strain R4(gfp) along the root surface; (E) strain R4(gfp)-‘infected’ plant cells of the root. Bars equal 30 µm.
843(gfp) (control strain) to see if root hair attachment could be linked to the ability to influence plant growth. Two hours after inoculation, attachment of the bacteria by one end to the surface of the root hairs (polar orientation) was observed in all of the tested strains (Figs 6A, B). More bacterial cells attached on root hairs inoculated with strain ANU843(gfp) (32.5 ± 9.2 (mean ± standard deviation) cells per 100 µm-length region of root hair) than root hairs inoculated with strain R4(gfp) (13.6 ± 4.9) or strain E4(gfp) (4.0 ± 2.5). Over the next 3–5 days the various *Rhizobium* strains increased their attachment to rice root hairs up to levels comparable to strain ANU843 (Fig. 6A).

**Root colonisation**

Fluorescence microscopy was used to compare the association of *Rhizobium* strain R4(gfp), which stimulated rice growth, strain E4(gfp), which inhibited rice growth, and strain ANU843(gfp) (as control) with rice roots. Three-day old rice seedlings of cv. Pelde were inoculated with tagged bacteria and grown in liquid medium in McCartney bottles, and colonisation studied at different times after inoculation.

*At day 3.* All bacterial strains were observed along the root grooves and in microcolonies growing on the root surfaces of the first main root.

*At day 7.* Strain R4(gfp) bacteria were observed along the grooves of the main and lateral roots, at lateral root junctions and on root tips. In contrast, strain E4(gfp) cells were seen only along root grooves on tap roots and at lateral root junctions, but not on the root tips.

*At day 14.* Strain R4(gfp) colonised the outer epidermal cells of tap roots and lateral roots, and formed long lines of colonies inside lateral roots on 10% of the inoculated

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**Fig. 6.** Root hair colonisation of *Rhizobium* strains tagged with the green fluorescent protein (gfp). (A) Colonisation of a root and its root hairs after 3 d with strain ANU843(gfp); (B) close up of root hair colonisation after 2 h with strain R4(gfp). Bars equal (A) 125 µm and (B) 25 µm.
Fig. 7. Fluorescence microscopy was used to study root colonisation of *Rhizobium* strain R4(gfp). The cv. Pelde seedlings were inoculated with strain R4(gfp), grown in liquid medium, and examined 21 d after inoculation. (A) When strain R4(gfp) colonised the lateral roots, it formed long lines of cells inside some of the lateral roots; (B) these lines of cells magnified. Note that these two long lines of bacterial cells were connected at the region indicated by white arrow; (C) Cross sections of these lateral roots (60 µm thick) showed fluorescence (arrowed) from this strain colonising intercellular spaces in the cortex region. Bars equal (A) 400 µm, (B) 50 µm and (C) 50 µm.
Rice seedling–bacteria interactions

The number of lateral roots per plant that showed that this phenomena ranged between one to two roots per seedling. Cross sections of these lateral roots showed that this strain colonised intercellular spaces in the cortex region (Fig. 7C). In contrast, strain E4(gfp) bacteria were observed only along root grooves, and no colonisation of intercellular spaces was detected. Strain ANU843, which was used as the control strain in these experiments, also did not colonise the intercellular spaces of seedlings. Furthermore, no specific morphological changes of roots, such as root hair curling or formation of infection threads, were observed on the roots of rice plants inoculated with either of these strains.

Bacterial colonisation in root and lateral root tissues

To aid our study of the entry of bacteria into the rice tissues, root tips were removed from the 3-day-old rice seedlings of cv. Pelde. This procedure stimulated an increase of lateral roots by 1.5- to 2-fold, potentially providing more entry sites for the bacteria via ‘crack entry’. In addition, this procedure could assist the bacteria to enter root tissues at the wound generated by root tip removal. The procedure was performed due to the current debate on whether, and at what levels, bacteria enter into the roots of crop plants via a form of ‘crack entry’. This is believed to occur at the junction of the emerging lateral roots and the main tap roots (Rolfe et al. 1998a; Webster et al. 1998; M. McCully, pers. comm.).

Treated roots were inoculated with the strain R4(gfp) or strain E4(gfp), seedlings grown in liquid medium in McCartney bottles, then examined 21 days after inoculation. Strain R4(gfp) could colonise inside lateral root tissues as well as inside the cut main root. 26% of seedlings were colonised by strain R4(gfp) which formed long lines of bacteria inside lateral roots (Fig. 7A). The number of lateral roots per plant that showed this phenomena ranged between one to five roots per seedling. Thus, the percentage of colonisation inside lateral roots was higher than on plants which were uncut (10%). The lines of bacterial colonisation inside lateral roots were not joined to the lines of bacterial colonisation inside the main root which had been cut at the tip. Interestingly, strain R4(gfp) only colonised inside lateral roots of the first main root, but did not colonise inside lateral roots of the second, third, fourth and subsequent main roots. In marked contrast, strains E4(gfp) and ANU843 did not colonise the internal tissues of the seedlings’ lateral roots or the cut ends of the main roots.

When uncut inoculated rice seedlings were grown in Magenta jars and inoculated with either strain R4(gfp), strain E4(gfp) or strain ANU843(gfp), a different phenomenon was observed with both strains E4(gfp) and ANU843(gfp). In McCartney bottles, the seedlings inoculated with strains E4(gfp) or ANU843(gfp) were not observed to colonise the intercellular spaces of the seedling. However, in the Magenta jar system at 21 days after inoculation of uncut seedling roots, both strains E4(gfp) and ANU843(gfp) bacteria were observed along the surface grooves on main and lateral roots, at lateral root junctions, on root tips. In addition, in 20% of the seedlings, both strains E4(gfp) and ANU843(gfp) bacteria were observed colonising intercellular spaces of lateral roots and forming short lines of cells. The average number of lateral roots per plant that showed this phenomena was limited to one root per seedling. Strain R4(gfp) behaved as in the experiments using liquid medium in McCartney bottles, forming long lines of bacteria (shown in Fig. 7A) in the lateral roots of 31% of inoculated seedlings. The number of lateral roots per plant that showed this phenomena ranged between one and three roots per seedling.

Multiplication of the bacteria within rice leaves

An additional plant assay was developed to analyse if there was any possible relationship between the ability of the rice-associating bacteria to affect seedling growth, and its ability to survive and multiply within rice tissues. As the environment provided by the rice leaf can be easily used to study internal colonisation by bacteria, this assay measured the multiplication, movement and compatibility of the strains within rice tissues.

In this bioassay, bacterial cells were pressure-infiltrated into sections of the rice seedling leaf and viable bacterial counts were recorded every two days. Using the different IRRI and Rhizobium strains, as well as the rice cultivars Pelde and Calrose, we observed that the rhizobia and IRRI strains can survive and multiply within rice leaves. The results for cultivar Pelde show that after infiltration into the leaf the different Rhizobium strains could multiply for 8–10 generations over a 12–15-d period (Fig. 8A). Even strain E4, which normally inhibits rice seedling growth, multiplied very well within rice leaves. The number of viable strain ANU843 bacteria that could be recovered in both rice cultivars declined after 9-d growth in the leaf (Fig. 8A).

These differences in growth and viability within rice seedling leaves were most evident when the IRRI strains were pressure-infiltrated into the leaves (Fig. 8B). The rice growth-promoting strain R38-0 grew rapidly and was found to multiply for 10–12 generations over the 15-d period, while strain R58, which also can enhance rice seedling growth, increased for 8–9 generations. In contrast, both strains R53 (a rice seedling growth-inhibiting strain) and R38-T (a rice seedling growth-promoting strain) grow only slowly, and increase for 3–4 generations over the same time period. Analysis of the bacterial levels in the leaf areas adjacent to the infiltration region showed that the multiplying rhizobia and IRRI strains do not move out of the 2-cm strip containing the sites of the original leaf inoculation.

Discussion

Recently, Rhizobium bacteria and several unclassified soil bacterial strains have been isolated and shown to form poten-
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Patial beneficial associations within the roots of rice plants grown either in laboratory experiments, pot trials (Barraquio et al. 1997), or in rice fields (Yanni et al. 1997). The present study was initiated to adapt and develop a standard set of growth assay techniques, and to use these techniques to investigate the consequences of the interactions of these newly isolated bacteria with several rice cultivars. What was needed was a set of simple, reliable and robust assays to establish the genetic basis of these microbe–rice plant interactions. Recently, Hecht-Buchholz (1998) pointed out that the most relevant problems of biological nitrogen fixation with non-legumes require new technologies for the visualisation and evaluation of intercellular bacterial colonisation, entry, spread and the establishment of reproducible internal colonisation. \textit{Rhizobium} bacteria were tagged with DNA sequences expressing the green fluorescent protein (gfp), a non-destructive assay, to follow the bacterial association with the roots of plants grown under three different methods of incubation. The experiments reported here examined the effects of the inoculation of these new rice-associating strains on the growth of seedlings of several rice cultivars, and the extent of rice root colonisation.

**Responses of rice cultivars to bacterial inoculation**

Rice seedling inoculation experiments have revealed three sources of variation in the rates of growth for the inoculated plants. These were (1) differences between rice cultivars in their ability to respond to bacterial inoculation, (2) variation in the performance of the bacterial strains and (3) variations due to the growth medium used. Not all the \textit{Rhizobium} and IRRI strains behaved in the same manner. Some strains stimulated rice plant growth, others had little effect, while others actually inhibited seedling development. Furthermore, the negative plant-microbe interactions could be altered readily by changing the growth medium conditions used in the experiments. The basis of the modulation of the growth inhibition in Hoagland’s medium, observed with strains R53 and E4, was investigated by changing the various media compositions in an attempt to define the crucial constituents involved. Even though the NFM + 10 mM KNO$_3$ and the Hoagland’s medium are fully defined media, our studies indicated that the basis of the modulation of the growth inhibition was a complex interaction between the levels of Ca$^{2+}$, K$^+$ and phosphate ions and the form and level of nitrogen addition. Further experimentation will refine the precise levels required.

It should be noted that some factors responsible for the stimulation of rice plant growth can, if produced in excess, become inhibitory. Possible candidates for such factors would be the phytohormones produced by these various bacteria, particularly auxins (Costacurta and Vanderleyden 1995). The various growth media could exert their effect by altering bacterial hormone production and hence the resulting plant growth responses. It needs to be established what effect inoculating with different ratios of stimulatory to inhibitory bacteria has on the early stages of rice seedling development. Clearly, an analysis of the resident micro-flora of rice-growing areas could be very informative and provide important information on the initial seedling growth, final yields and grain nitrogen content.

**Visualisation of \textit{Rhizobium} colonisation of rice roots**

With the establishment of strain-dependent growth effects, several strains, such as strains R4 and E4, were
chosen to examine their interactions with rice roots more closely. The roots of rice plants grown on agar plates did not generally have an extensive bacterial colonisation, and where it occurred, the bacteria were observed in clumps along the developing root. Bacteria were observed along the grooves of the main roots and in microcolonies on the root surfaces, often at the junction of a lateral root. Polar orientation of bacterial attachment was observed for all of the test strains. This was similar to that observed for *R. l. trifolii* strain 4S (Terouchi and Syono 1990), *Azorhizobium caulinodans* strain ORS571 on rice roots (Reddy *et al.* 1997) and *Rhizobium trifolii* on clovers (Dazzo *et al.* 1984). Root hair curling was not observed, as usually seen on host legumes, or as reported for monocot oat seedlings where the root hairs were reported to curl 2 d after inoculation with *R. l. trifolii* strain 4S (Terouchi and Syono 1990). However, there were examples where the bacteria were located in host cells, hence some type of entry had obviously taken place. This concentration of bacteria could have been due to accumulation or possibly some multiplication inside the root cells, and might be considered endophytic since microscopy showed that it occurred inside the root beneath the epidermis.

A different picture emerged when colonisation studies were performed with seedlings growing in liquid medium in McCartney bottles. Two strains, R4 and E4, were investigated because of the different growth responses they produce on rice cultivar Pelde. Strain R4 entered into lateral roots in 10% of the rice seedlings, and formed long lines of cells along the lateral root as it grew. This frequency of lateral root occupancy could be increased up to 26% of the plants if the tip of the main root was first removed before bacterial inoculation. Strikingly, *Rhizobium* strain E4 was not observed to enter into rice root tissues, but only to colonise the root surfaces.

When the inoculated seedlings were grown in liquid medium in Magenta jars, however, the differences between the two strains R4(gfp) and E4(gfp) were greatly reduced, and the control strain ANU843(gfp) also entered roots at the same level as the E4(gfp) strain. All three strains could enter the roots and form intercellular lines of bacterial colonisation in growing lateral roots, although strain R4(gfp) did so at a higher frequency. The rice plants grew more vigorously with extensive root formation in Magenta jars. The potential gas exchange (calculated as liquid surface area to liquid volume ratio) for both liquid medium conditions was not significantly different. Thus, it is likely that the physical dimensions of the assay container affect the extensiveness of the rice root growth, and that this in turn plays a major role in the observed association of the bacterial strains with the growing roots and the intimacy of this interaction. These results show no linkage between the ability of a strain to colonise inside a lateral root and its ability to alter seedling growth. However, the development of this assay technique will be valuable in future experiments to establish the molecular basis of the influence of rice roots on bacterial colonisation.

In summary, the experiments with gfp-tagged *Rhizobium* strains indicate that these bacteria behave more like intimate epiphytic microbes (Dickinson and Lucas 1982) and multiply on the plant surfaces without producing any obvious disease effects, but still provide a substantial source of stimulatory molecules for plant growth. Some of the bacterial strains examined can behave as endophytes, entering and becoming intercellular in rice tissues, while other strains have not yet been shown to do so. However, some caution is warranted with these assessments, as the physical growth conditions can markedly influence the extent of the observed interactions and the subsequent characterisation of a particular strain.

**Growth of bacteria in rice leaves**

In the absence of a reproducible method of introducing bacteria inside rice roots, a rice leaf growth assay was developed to study the internal colonisation of the endophytic strains by measuring their multiplication, movement and compatibility within leaf tissues. This assay also enables the use of various bacterial strains to be used as biological ‘probes’ of any induced responses or preformed systems of plant responses in the rice plants. The *hrp* genes and their associated type III secretion system are thought not to be ubiquitous in rhizobia (Viprey *et al.* 1998), but appear to be important in the symbiotic interactions of *Rhizobium* sp. NGR234 and its nodulation of some tropical legumes. The type III secretory systems are essential components of both animal and plant pathogens and their interactions with eukaryotic cells (Viprey *et al.* 1998). Hence, these rice leaf growth assays were carried out to make an assessment of the individual strains and to aid in their further characterisation as internal colonists.

The results showed that there were differences in the extent of growth between strains. These variations in bacterial numbers therefore represent considerable differences in survival within the rice tissues. These findings suggest that some of the endophytic isolates used, such as strain R38-0, possess important traits that enhance their competitive ability to colonise internal niches within rice tissues. However, IRRI strain R53, which inhibited plant growth, grew poorly within rice leaves, and strain R38-T, which could stimulate seedling growth, also grew slowly in rice seedling leaves.

Bacterial assays to examine the spread of the bacteria within the leaf tissues away from the sites of the original leaf inoculation region found that even *Rhizobium* strains, which can form long lines of internal root colonisation, do not move out of the 2-cm leaf inoculation site. We have shown that these bacteria can multiply both within the leaf and the lateral roots. However, it is only in the lateral roots where we have seen evidence of bacterial migration. This surprising result indicates that we will need to continue the development of specific root assays to assess the capacities of these strains to spread within the roots once entry has been gained.
The general conclusion is that a number of the bacterial strains tested, *Rhizobium* strain R4 and IRRI strains R38-0 and R38-T, can significantly alter the growth of rice seedlings, and that many of these strains also have the ability to colonise and survive inside the rice seedling tissues. As some strains possess the ability to colonise without altering seedling growth, it is possible that the genes responsible for the colonisation ability are separate from those genes responsible for the effects on seedling growth. By adapting and developing techniques to reliably assess the performance of rice-associating strains in their promotion of rice seedling growth and growth in rice leaves, we can now begin to use molecular genetic techniques to identify the genes in these bacteria which are responsible for these rice-associative characteristics.

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