THE ROYAL SOCIETY

The xylem of rice (*Oryza sativa*) is colonized by *Azorhizobium caulinodans*

Ganesan Gopalaswamy[1], Sadasivam Kannaiyan[1], Kenneth J. O'Callaghan[2], Michael R. Davey[2] and Edward C. Cocking[2*]

[1]*Tamilnadu Agricultural University, Coimbatore-641003, Tamilnadu, India*
[2]*Centre for Crop Nitrogen Fixation, Plant Science Division, School of Biological Sciences, University of Nottingham, Nottingham NG7 2RD, UK*

Following inoculation with *Azorhizobium caulinodans* ORS571(pXLGD4), lateral root development of rice and colonization of lateral root cracks by bacteria were shown to be stimulated by the flavonoid naringenin. Rice seedlings growing aseptically in the presence of naringenin were inoculated with ORS571(pXLGD4), carrying the *lac*Z reporter gene. By microscopic analysis of sections of inoculated rice roots, it has been demonstrated that the xylem of rice roots can be colonized by *Azorhizobium caulinodans*. We discuss whether this colonization of the xylem of rice roots by azorhizobia could provide a suitable niche for endophytic nitrogen fixation.

Keywords: *Azorhizobium caulinodans*; crack entry; lateral roots; naringenin; rice; xylem colonization

1. INTRODUCTION

Our finding that the diazotroph *Azorhizobium caulinodans* ORS571 colonizes the xylem of roots of the legume *Sesbania rostrata* (O'Callaghan *et al*. 1997) in addition to colonizing nodules in that legume, led us to investigate whether this rhizobial strain, whilst unable to nodulate rice, might nevertheless be able to colonize the xylem of rice roots. Colonization of the xylem of rice by *A. caulinodans* ORS571 is of interest because it might provide a suitable nutritional and environmental niche for nitrogen fixation, comparable to the situation in sugar cane in which endophytic diazotrophs internally colonize the plant, including the xylem, and are probably major contributors to the observed high levels of nitrogen fixation (Boddey 1995). Our previous studies on rice grown in the presence of the flavonoid naringenin and inoculated with ORS571 showed entry of bacteria at lateral root cracks (LRCs) and the presence of large intercellular pockets of azorhizobia in the cortex. However, xylem colonization was not observed (Webster *et al*. 1997). Reddy *et al*. (1997) have also observed that ORS571 enters rice roots through cracks in the epidermis created during emergence of lateral roots and that azorhizobia invade intercellularly the outer cortex. Again, xylem colonization was not detected.

In the present study, we have performed microscopic analyses of sections of the junction regions of lateral and primary roots of rice inoculated in the presence of naringenin with ORS571 (pXLGD4) carrying the *lac*Z reporter gene. We have demonstrated for the first time, to our knowledge, that the xylem of rice roots is colonized by *A. caulinodans*.

2. MATERIAL AND METHODS

(a) *Plant growth conditions and inoculation*

Rice (*Oryza sativa*) seeds (the American cv. Lemont and the Indian cvs ADT36, CO43 and CR1009) were dehusked, surface sterilized using 30% (v/v) 'Domestos' bleach (Lever Industrial Ltd, Runcorn, UK) for 60 min, washed five times in sterile distilled water and germinated aseptically for 24 h in the dark (28 °C) on 0.8% (w/v) agar (Sigma, Poole, UK). Germinated seeds were transferred aseptically to tubes (25 mm × 200 mm, 70 ml capacity), each containing 20 ml nitrogen-free medium (Fåhraeus, 1957) semi-solidified with 0.8% (w/v) agar, either with or without naringenin (Sigma) at 5×10^{-5} M. The flavonoid naringenin is the aglucon of naringin, which is found in the flowers and fruit of grapefruit trees. After four days' growth in tubes, seedlings were each inoculated with 0.5 ml (10^8 ml^{-1}) of either *A. caulinodans* ORS571 (pXLGD4) cultured in TGYE liquid medium (Somasegaran & Hoben 1994), or *Azospirillum brasilense* SP7 (pLA-*lac*Z) cultured in YEP liquid medium (Vanstockem *et al*. 1987); uninoculated plants received 0.5 ml of either TGYE or YEP medium. Plants were incubated at 28 °C (14 h photoperiod; 250 μEm^{-2} s^{-1} 'daylight' fluorescent tubes) for 21 days. Surface-sterilized pre-germinated rice seeds (cv. CO43) were also grown in sterilized plastic pots in a sterile vermiculite–perlite mixture (1:1, v/v) (200 g per pot) under similar growth-room conditions. Plants were watered daily with sterile nitrogen-free Fåhraeus medium. Inoculation of each plant with 2 ml *A. caulinodans* ORS571 (pXLGD4) (10^8 ml^{-1}), with 5×10^{-5} M naringenin, was at planting and every seven days thereafter; uninoculated plants each similarly received 2 ml TGYE medium with 5×10^{-5} M naringenin.

(b) *Detection of bacterial β-galactosidase activity*

Bacterial colonization of the roots of inoculated plants was visualized by light microscopy of the dark blue precipitate resulting from the degradation of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) by β-galactosidase (Arsène *et al*. 1994). Seedlings were fixed in 2% (v/v) glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.2) for 2.5 h in order to inactivate endogenous plant β-galactosidase activity (Teeri *et al*. 1989), prior to thorough rinsing in buffer only and incubation with X-Gal (Boivin *et al*. 1990).

(c) *Microscopic procedures*

Root pieces of interest exhibiting blue histochemical precipitate were excised intact. Specimens fixed in 2% glutaraldehyde

*Author for correspondence (edward.cocking@nottingham.ac.uk).

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(v/v) in sodium cacodylate buffer (pH 7.2) were dehydrated through a graded ethanol series and embedded in LR White resin (The London Resin Co., Basingstoke, UK). Sections for light microscopy (1 µm thick) were observed either with or without staining with 0.5% (w/v) toluidine blue in sodium tetraborate (Davey *et al.* 1993). Ultrastructural observations were performed on tissue samples fixed and embedded as described for light microscopy; ultrathin sections were post-stained also as previously described (O'Callaghan *et al.* 1997).

(d) *Reisolation of ORS571 (pXLGD4) from inoculated rice*

Roots from 21-day-old seedlings (cv. ADT36) previously inoculated with *A. caulinodans* ORS571 (pXLGD4), and from uninoculated seedlings (cv. ADT36) of the same age, were surface sterilized as described previously and macerated with sterile sand and water. Extracts were serially diluted and cultured in semi-solidified (0.8% w/v agar) TGYE medium containing 0.5% (v/v) of a solution of 2% (w/v) X-Gal in dimethyl formamide to estimate the number of *A. caulinodans* ORS571 (pXLGD4) present within the surface-sterilized roots.

3. RESULTS

Root development was investigated in the four rice varieties, Lemont, C043, ADT36 and CR1009 inoculated with *A. caulinodans* ORS571 (pXLGD4) and grown for three weeks in tubes under gnotobiotic conditions. The number of lateral roots, including thick short lateral roots, on seedlings of CR1009 inoculated five days after germination with different quantities of ORS571 (pXLGD4) is given in table 1. The presence of 5×10^{-5} M naringenin increased significantly the number of laterals on the seminal and adventitious roots of the variety CR1009 (table 1) and also on the other three rice varieties assessed (data not shown). The effect of naringenin on the number of lateral roots in the same four rice varieties inoculated under comparable conditions with *Azospirillum brasilense* SP7 (pLA-*lac*Z) was also investigated. The presence of 5×10^{-5} M naringenin also increased significantly the number of laterals on seminal and adventitious roots in all the four rice varieties (data not shown).

Colonization of LRCs by ORS571 (pXLGD4) and any nearby intercellular colonization of the cortex, as visualized using X-Gal, were grouped together as LRC colonization, since they can be considered as different stages of the same process for purposes of quantification of LRC colonization. The number of colonized LRCs per plant was counted ($n=5$) and expressed as a percentage of the total number of LRCs per plant (a mean percentage of LRCs colonized per plant). In all four 26-day-old rice varieties, the presence of 5×10^{-5} M naringenin significantly increased the mean percentage of LRCs colonized per plant (table 2). Inoculation with *Azospirillum* SP7 (pLA-*lac*Z) under comparable conditions in the presence of 5×10^{-5} M naringenin also resulted in a significant increase in the mean percentage of the LRCs colonized per plant in all four rice varieties (data not shown).

Sections of roots of tube-grown plants of the rice varieties ADT36 and CR1009 inoculated with *A. caulinodans* (pXLGD4), in the presence of 5×10^{-5} M naringenin, were examined by light and electron microscopy. Following X-Gal treatment, bacteria expressing *lac*Z could be readily located histochemically since they produced a dark blue precipitate. Crack entry colonization was visible in sections of dark blue regions excised from the bases of lateral roots; deep-seated intercellular colonization of the cortex was also detected (figure 1*a*). Light microscopy (using oil immersion) of sections of these dark blue regions from the bases of lateral roots revealed the presence of bacteria, expressing *lac*Z in the xylem at the base of lateral roots in approximately half of the dark blue regions from the bases of lateral roots examined (figure 1*b*). Xylem colonization was observed in toluidine blue stained similar sections (figure 1*c*) and in toluidine blue stained sections of the xylem of the primary roots opposite to the base of lateral roots (figure 1*d*). Electron microscopy verified the presence of bacteria in the xylem at the base of lateral roots (figure 1*e*) where *A. caulinodans* ORS571 (pXLGD4) had been detected by light microscopy, following X-Gal treatment (figure 1*b*). Xylem colonization was not observed in sections of dark blue regions from the bases of lateral roots of rice inoculated similarly with *A. caulinodans*

Table 1. *Effect of naringenin on the number of lateral roots of* Oryza sativa *(cv. CR1009) inoculated with different quantities of* Azorhizobium caulinodans *ORS571 (pXLGD4)*

(For each level of inoculation the presence of naringenin (5×10^{-5}M) caused a significant increase in lateral root numbers on seminal and adventitious roots at the $p=0.01$ level.)

	number of laterals per plant (±s.d.)	
inoculation	seminal root	adventitious roots
0.2 ml	97.8±9.9	106.4±11.2
0.2 ml + naringenin	135.0±10.1	232.6±38.7
0.5 ml	109.2±4.6	125.0±13.7
0.5 ml + naringenin	141.8±12.6	254.2±34.4
1.0 ml	90.4±6.8	111.0±17.5
1.0 ml + naringenin	115.0±10.4	229.4±17.9

Table 2. *Effect of naringenin on LRC colonization of four varieties of* Oryza sativa *inoculated with 0.5 ml* Azorhizobium caulinodans *ORS571 (pXLGD4)*

(For any variety the presence of naringenin (5×10^{-5}M) caused a significant increase in the mean percentage of LRCs colonized per plant ($n=5$) at the $p=0.01$ level.)

		percentage of LRCs colonized per plant (±s.d.)	
variety	treatment	seminal root	adventitious roots
Lemont	—	9.4±0.7	5.7±1.2
	+ naringenin	20.9±1.5	10.7±0.6
CO43	—	8.3±0.6	11.0±0.4
	+ naringenin	10.2±1.3	16.1±1.0
ADT36	—	6.7±1.1	14.5±1.5
	+ naringenin	9.3±0.6	37.6±3.1
CR1009	—	5.3±0.6	11.3±1.1
	+ naringenin	8.8±0.9	16.5±1.2

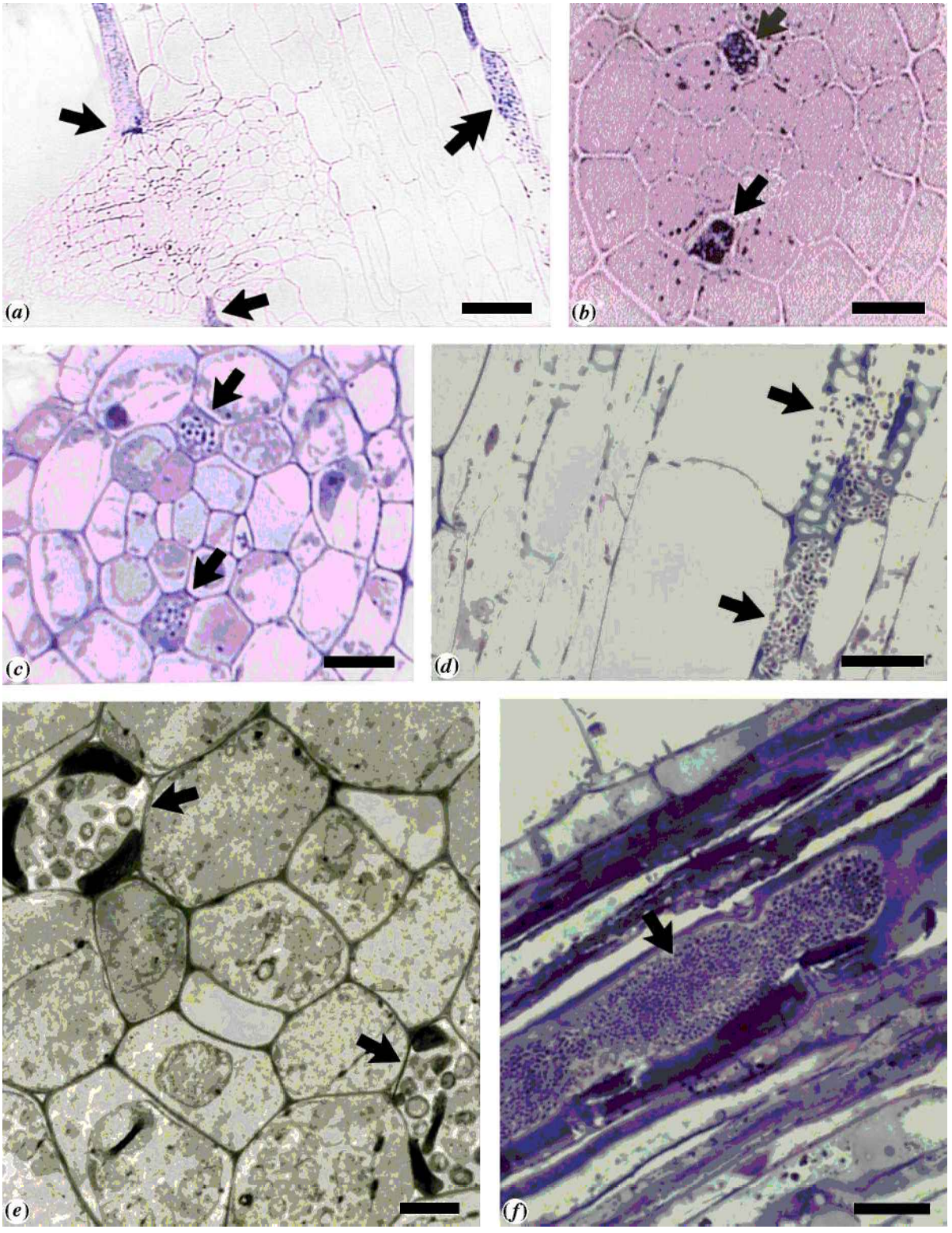

Figure 1. Light (*a–d, f*) and electron micrographs (*e*) of colonization of rice following inoculation with *Azorhizobium caulinodans* ORS571 (pXLGD4). (*a*) LRC colonization sites (arrows) and deep-seated intercellular colonization of the cortex (double arrow) (cv. CR1009) visualized by histochemical staining (dark blue colour) of bacterial β-galactosidase activity. (*b*) Dark blue precipitate in the xylem (arrow) of a transverse section of a lateral root (cv. ADT36) following histochemical staining of bacterial β-galactosidase activity (5×10^{-5}M naringenin). (*c*) Transverse section of a lateral root stained with toluidine blue as in (*b*) (xylem colonization, arrows). (*d*) Longitudinal section of a primary root (cv. CR1009) stained with toluidine blue showing colonized xylem (arrows). (*e*) Electron micrograph of a lateral root as in (*b*) and (*c*) showing bacteria in xylem elements (arrows). (*f*) Longitudinal section of a primary root (cv. CO43) stained with toluidine blue showing extensive colonization of the xylem (arrow) (5×10^{-5} M naringenin). Scale bars represent (*a*) 100 µm, (*b–d, f*) 10 µm and (*e*) 2 µm.

ORS571 (pXLDG4), but in the absence of naringenin. Approximately 10^5 ORS571 (pXLGD4) per root system were re-isolated from surface-sterilized roots of inoculated plants of the variety ADT36 grown for 21 days in tubes with 5×10^{-5} M naringenin; bacteria could not be re-isolated from uninoculated plants grown similarly. Sections revealed extensive colonization of the xylem of primary roots of rice (variety CO43) grown for 21 days in pots under controlled growth-room conditions, and inoculated repeatedly with *A. caulinodans* ORS571 (pXLGD4) and 5×10^{-5} M naringenin (figure 1*f*). Bacteria could not be found in toluidine blue stained sections of primary or lateral roots of uninoculated rice plants ($n=15$) grown in pots under similar conditions.

Sections of roots of tube-grown plants of the rice varieties ADT36 and CR1009 were also examined following inoculation with *Azospirillum* SP7 (pLA-*lac*Z) under similar conditions in the presence of 5×10^{-5} M naringenin. Crack entry colonization was detected following X-Gal treatment, together with extensive surface colonization of roots. However, there was no deep-seated intercellular colonization of the cortex and no colonization of the xylem of the roots of these plants.

4. DISCUSSION

A large number of plant species exhibit xylem colonization by bacteria without disease symptoms and xylem colonization is increasingly being seen as a common aspect of plant–microbe interactions (Hallmann *et al*. 1997). Moreover, it is becoming increasingly realized that the xylem may be more robust structurally and physiologically than previously envisaged and that it should no longer be regarded as a vulnerable pipeline on the edge of disaster (Canny 1998). The non-rhizobial diazotroph, *Acetobacter diazotrophicus* has been shown to penetrate sugar cane roots intercellularly at the root tip and at cracks in lateral root junctions and to colonize xylem vessels following the inoculation of aseptically grown plants (James *et al*. 1994). Little is known, however, as to how exactly bacteria reach and invade the xylem. It has been suggested that xylem elements are possible sites of nitrogen fixation by diazotrophs, since the xylem elements could provide the low pO_2 and a site for exchange of metabolites necessary for nitrogen fixation (James *et al*. 1994; Rolfe *et al*. 1998). Since *A. caulinodans* ORS571 is able to fix nitrogen in the free-living state without differentiation into bacteroids in up to 3% oxygen (Kitts & Ludwig 1994), this first report of the colonization of xylem elements of rice by azorhizobia may be of significance in this respect. The pO_2 in rice roots is likely to be influenced by waterlogging of the rice plant and more extensive invasion will probably be required to have an impact on the nitrogen balance.

The non-rhizobial diazotroph *Xanthobacter* spp. are closely related to *A. caulinodans* ORS571 (Rainey & Wiegel 1996), and a rice isolate of *Xanthobacter* has been shown to increase plant growth (Wiegel 1992). Another non-rhizobial diazotroph, *Azoarcus* sp. BH72, was found to ingress intercellularly into the stele of kallar grass, in gnotobiotic culture, and was also detected in the xylem. In gnotobiotic cultures, the xylem of rice plants was also infected by *Azoarcus* sp. BH72 (Hurek *et al*. 1994). A study of pathogenic interactions between tomato roots and *Ralstonia solanacearum* showed that these bacteria infect intercellularly the inner cortex and protoxylem vessels. In order to invade the vascular cylinder, *R. solanacearum* bacteria target sites where the endodermis is either incompletely differentiated at root extremities, or is reorientated at secondary root axils by the outgrowth and development of lateral roots (Vasse *et al*. 1995; Etchebar *et al*. 1998). It seems likely that *A. caulinodans* ORS571, in its pathway to xylem invasion, also exploits sites where the physical endodermal barrier is reorientated at secondary root axils by the outgrowth and development of lateral roots. ORS571, in addition to inducing and invading nodules in the root cortex, is known to colonize xylem elements of the legume *Sesbania rostrata* (O'Callaghan *et al*. 1997). In the present study, the ability to detect xylem colonization by azorhizobia at lateral root junctions has been facilitated by the use of the *lac*Z reporter gene to identify regions of roots colonized extensively by azorhizobia. Such isolated dark blue regions may be more likely to exhibit xylem colonization.

The non-rhizobial diazotroph *Azospirillum brasilense* SP7 has been shown to enhance the formation of lateral roots of wheat (Bothe *et al*. 1992). *Azospirillum brasilense* SP7 and SP245 are able to colonize LRCs of wheat (Webster *et al*. 1998) and *Arabidopsis* (Gough *et al*. 1997), with LRC colonization being stimulated significantly by naringenin. However, azospirilla could not be detected in root xylem, but were detected in intercellular spaces in the cortex of wheat plants inoculated with SP245 (Assmus *et al*. 1995).

The extensive colonization by *A. caulinodans* of the xylem of the roots of rice grown in pots is reminiscent of the invasion of the xylem of lateral roots of wheat inoculated with *A. caulinodans* and grown in pots under controlled conditions (Sabry *et al*. 1997). The addition of naringenin may enhance xylem colonization by azorhizobia as a consequence of its stimulatory effect on lateral root development and on LRC colonization. However, there is also the possibility that the general invasiveness of *A. caulinodans* may be enhanced by this flavonoid. Flavonoids have been shown to induce resistance in *Bradyrhizobium japonicum* and *Rhizobium fredii* to the soya bean phytoalexin, glyceollin (Parniske *et al*. 1991). There is thus the possibility that the addition of naringenin may increase the tolerance of inoculated azorhizobia to toxic compounds secreted by rice roots. It was suggested previously that xylem colonization of *Arabidopsis thaliana* by ORS571 might necessitate the transfer of the ability to colonize xylem from the plant pathogen *R. solanacearum* through the integration of a gene library of *R. solanacearum* into ORS571 (Gough *et al*. 1997). However, the present results demonstrate that this is not necessary for xylem colonization of rice by *A. caulinodans* ORS571.

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