Xylem colonization of the legume *Sesbania rostrata* by *Azorhizobium caulinodans*

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SUMMARY

A novel pathway of invasion of the legume *Sesbania rostrata* by *Azorhizobium caulinodans* is described that involves colonization of the root xylem, possibly following entry into the natural fissures created during emergence of lateral roots. Azorhizobia were detected microscopically, and their presence confirmed by the expression of a *lac*Z reporter gene. We have shown that rhizobial Nod factors are not required for either xylem colonization or for crack-entry of lateral roots. We discuss the extent to which this discovery of xylem colonization by azorhizobia is likely to improve our understanding of both symbiosis and of pathogenicity in plant–bacterial interactions.

1. INTRODUCTION

Rhizobia are bacteria that invade legumes after activating cell division in the plant root cortex, initiating a new organ, the nitrogen-fixing nodule (Long 1996). Since the nodule is generally considered the only endophytic destination of invading rhizobia, most previous studies have focused on the rhizobial invasion pathway into and within the cortex (Kijne 1992). Many non-rhizobial endophytic bacteria colonize the vascular system of plants, sometimes, but not always leading to plant disease (Bell *et al.* 1995). For example, the xylem of healthy alfalfa, a legume, is colonized by non-rhizobial endophytes (Gagné *et al.* 1987). Agrobacteria, plant pathogens closely related taxonomically to rhizobia, invade the xylem of several species including, interestingly, the tropical legume *Sesbania rostrata* (Vlachova *et al.* 1987). Inoculation of *S. rostrata* with *Azorhizobium caulinodans* ORS571 (Dreyfus *et al.* 1988) results in production of nodules on roots (Ndoye *et al.* 1994) and stems (Tsien *et al.* 1983). Interestingly, ORS571 is able to establish itself endophytically in the roots of rice (Christiansen-Weniger 1996) and wheat (Sabry *et al.* 1997), although these plants are not natural hosts and nodules do not develop.

In our study, we have found that ORS571 colonizes xylem elements, in addition to inducing and invading nodules in the root cortex of *S. rostrata*. However, we have demonstrated that xylem colonization is not regulated in the same way as nodulation. Thus, for the first time, a species of legume nodule bacteria has been found to colonize, reproducibly, regions of the host plant other than nodules. This novel endophytic interaction will be of interest to phytopathologists, to researchers investigating legume–rhizobia interactions, and to workers attempting to extend endophytic rhizobial nitrogen fixation to non-legumes.

2. MATERIALS AND METHODS

(*a*) *Culture of rhizobia*

All rhizobia were cultured on media semi-solidified with 0.8% (w/v) agar. *Azorhizobium caulinodans* ORS571 was supplied by Dr D. Geelen (Gent), and cultured on TY medium (Somasegaran & Hoben 1994). Five strains carrying pXLGD4, containing the *lac*Z reporter gene, were supplied by J. Dénarié (INRA-CNRS, Toulouse, France). These pXLGD4-containing strains were cultured and selected as follows. ORS571 (pXLGD4), ORS571::*nodC* (pXLGD4) and *Rhizobium meliloti* RCR2011 (pXLGD4) were cultured on TY medium with 10 μg ml^{-1} of tetracyclin. The medium for the *nodC* strain also contained 25 μg ml^{-1} of kanamycin. *Sinorhizobium saheli* ORS611 (pXLGD4) and *S. teranga* ORS604 (pXLGD4) were cultured on YM medium (Somasegaran & Hoben 1994) with 100 μg ml^{-1} of streptomycin and 10 μg ml^{-1} of tetracyclin. Bacteria re-isolated from macerated tissue of surface-sterilized, tube-grown plants of *Sesbania rostrata* were plated onto TY medium, TY medium containing Congo Red (Somasegaran & Hoben 1994), and TY medium containing kanamycin and tetracyclin with 0.1 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The latter was added as a 2% (w/v) solution in dimethyl formamide. ORS571, ORS571 (pXLGD4), *S. saheli* and *S. teranga* effectively nodulated *S. rostrata* and fixed nitrogen as assessed using the acetylene reduction assay (data not shown).

(*b*) *Plant material and inoculation procedures*

Sesbania rostrata seeds (Dr J. K. Ladha, International Rice Research Institute (IRRI)) were scarified by immersion in hot water (*ca.* 100 °C), and left to cool; seeds were removed after 5 h. Seeds were surface-sterilized in 10% (v/v) 'Domestos' bleach (Lever Industrial Ltd, Runcorn, UK) for 10 min, rinsed thoroughly in sterile water and germinated on 0.8% (w/v) water agar plates for one day in the dark at 28 °C. Seedlings were transferred aseptically to sterile tubes (2.5 × 15 cm), each containing 25 ml of nitrogen-free medium (Fåhraeus 1957), and placed under Daylight fluorescent tubes

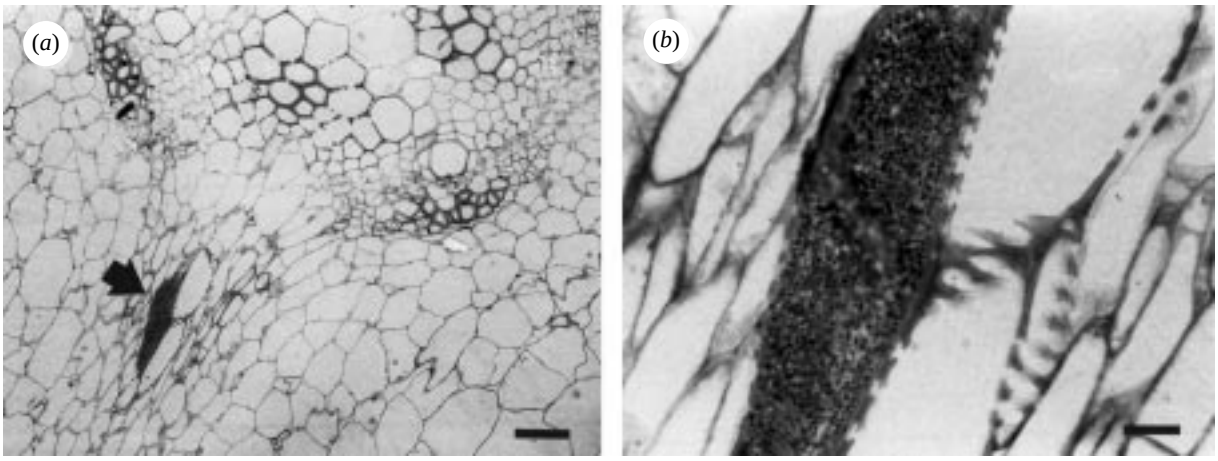

Figure 1. Bright field photomicrographs of sections of the lateral root of a two-week old plant of *S. rostrata* inoculated with *Azorhizobium caulinodans* ORS571. (*a*) Toluidine blue-stained section through a primary root at a lateral root junction, showing bacteria (arrowed) in a xylem element of the lateral. Scale bar, 127 μm. (*b*) Higher magnification of (*a*), showing a xylem element presumed to contain ORS571. Scale bar, 25 μm.

(37 μE m^{-2} s^{-1} illuminance) in a growth room (25 °C day, 22 °C night) with a 16-hour photoperiod. After one day, seedlings were inoculated with either 200 μl of a suspension of rhizobia in sterile distilled water at a density of *ca.* 10^8 bacteria ml^{-1}, or with 200 μl of sterile distilled water only. Plants were examined after 2–4 weeks and prepared for microscopy and *lacZ* assay. Additionally, one-day old seedlings (germinated as described) were also placed in black plastic bags of 0.64 l capacity (10 × 8 × 8 cm) containing a mixture of vermiculite and perlite (1:1, v:v) and grown in the glasshouse under natural daylight (maximum day temperature 28 °C; minimum night temperature 18 °C). After one week, seedlings were inoculated either with 2 ml of azorhizobia, or with water as previously described. Seeds of *Aeschynomene afraspera* and *A. nilotica* (both supplied by Dr J. K. Ladha) were germinated and inoculated, and seedlings were examined in the same way as tube-grown plants of *S. rostrata*.

(*c*) LacZ *reporter gene assays*

In order to confirm, quantify and examine xylem colonization by ORS571, plants of *S. rostrata* were inoculated with rhizobial strains carrying pXLGD4, containing a constitutive *lacZ* reporter gene. This gene confers β-galactosidase activity, which enables bacteria to be localized by the blue precipitate produced after degradation of the chromogenic substrate X-Gal. Tube and pot-grown seedlings were inoculated one and seven days after germination, and examined at 2–4 and 8–10 weeks, respectively. Intact root systems were excised from plants and treated with X-Gal as described (Boivin *et al.* 1990), except that the roots were fixed for 2 h at atmospheric pressure. Primary roots of tube-grown plants were sectioned transversely by hand into a series of explants each *ca.* 0.5 mm thick; lateral roots present in these sections were examined microscopically for the presence of blue precipitate. Pot-grown plants were relatively large and woody; consequently, only pieces of recently formed tertiary root were examined.

In order to determine whether any bacteria in suspension during the X-Gal reaction procedure, which includes various liquid infiltration stages, were entrained into the xylem causing a spurious colonization, root systems from uninoculated plants were placed in 0.1 M sodium phosphate buffer (pH 7.0) containing *ca.* 2.5×10^8 ORS571(pXLGD4) bacteria ml^{-1}. After 1.5 h, roots received the standard X-Gal treatment, and were sectioned by hand as described.

(*d*) *Preparation of material for light and electron microscopy*

Plants were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0). Root explants were processed for light and electron microscopy as described previously (Davey *et al.* 1993).

3. RESULTS

(*a*) *Xylem colonization of* Sesbania

Toluidine blue-stained sections of material from plants inoculated with ORS571 showed numerous bacteria in the xylem of emergent lateral roots (figure 1*a*,*b*), with electron microscopy confirming the presence of bacteria in the xylem elements (figure 2*a*). Bacteria were not observed in more than 25 similar sections from each of two uninoculated tube-grown plants. The *lacZ* procedure included glutaraldehyde prefixation, which virtually eliminated endogenous plant β-galactosidase activity. This allowed bacteria expressing *lacZ* to be localized easily, as they produced a dark blue precipitate. Examination of hand-cut transverse sections of primary roots of tube-grown plants inoculated with ORS571(pXLGD4), revealed a linear band of blue precipitate in the vascular system of emergent lateral roots (figure 3*a*) of approximately 27% of these plants. A plant was recorded as having invaded xylem when any laterals showed a straight continuous blue band spanning more than half the distance between the pericycle and the epidermis of the primary root. Blue bands were not seen in laterals of uninoculated plants treated with X-Gal. In further controls, roots from uninoculated plants were placed in sodium phosphate buffer containing ORS571(pXLGD4) bacteria, but after X-Gal treatment, blue bands were not seen in the xylem, confirming that the bands seen in inoculated plants were not artefacts arising from the *lacZ* assay procedure. Examination of thin sections of blue bands at higher magnification showed that the blue precipitate was within the xylem (figure 3*b*). Electron microscopy confirmed the presence of numerous bacteria in xylem elements of lateral roots which contained blue bands

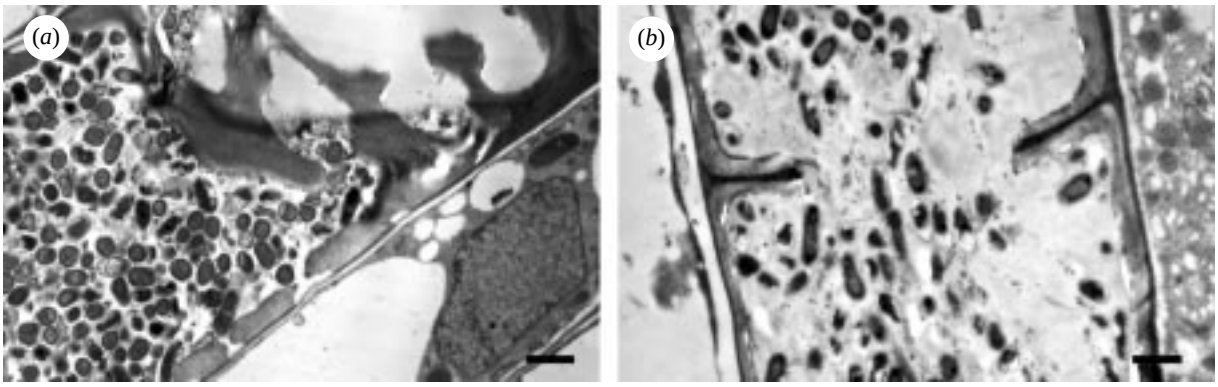

Figure 2. Electron micrographs showing azorhizobia in the xylem elements of lateral roots of *S. rostrata* inoculated with *A. caulinodans*. (*a*) Inoculated with ORS571; material as in figure 1. Scale bar, 2 μm (*b*) Inoculated with ORS571(pXLGD4); material as in figure 3*a*. Scale bar, 1.6 μm.

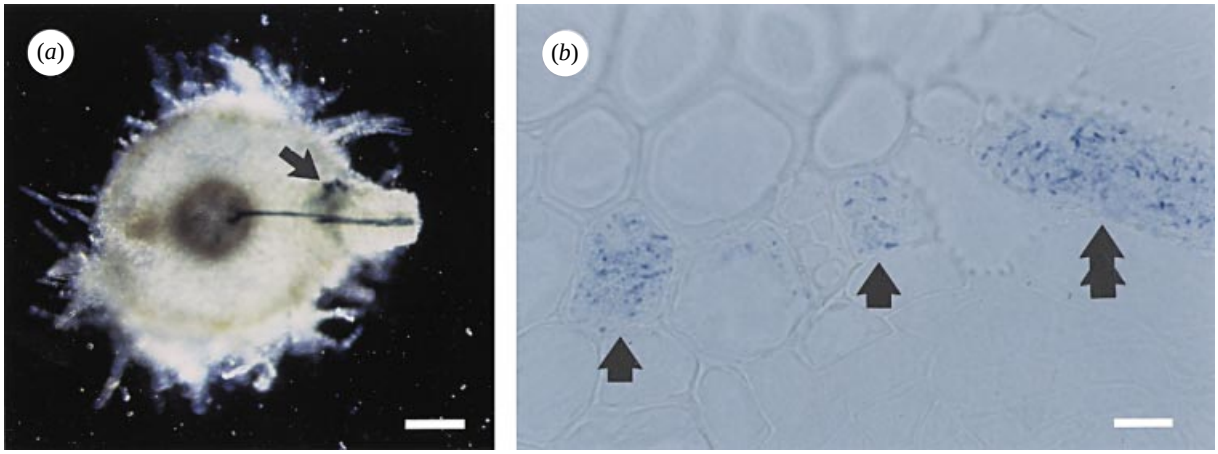

Figure 3. Photomicrographs of sections of a primary root at a lateral root junction of a two-week old plant of *S. rostrata* inoculated with ORS571(pXLGD4). (*a*) Hand-cut section showing a blue linear band of precipitate in the vascular system of the emergent lateral root. Blue precipitate at the point of crack entry (arrowed) is also visible. Scale bar, 0.3 mm. (*b*) Higher magnification of embedded and sectioned material showing blue staining bacteria within xylem elements of both the primary root (single arrows) and the lateral root (double arrow). Scale bars, 25 μm.

(figure 2*b*). In general, these blue bands were found in lateral roots lacking nodules. The pot-grown plants had thick, lignified primary roots, but the younger, tertiary roots were relatively translucent, and some of these roots had blue precipitate within their vascular tissue. Blue precipitate was observed in the xylem when thin longitudinal sections of excised pieces of these tertiary roots were examined at high magnification by light microscopy. Again, electron microscopy verified the presence of bacteria in the xylem, in those regions where the blue precipitate had been observed by light microscopy (figures not shown).

(*b*) *Host range studies*

In order to examine the host plant range of ORS571 with respect to xylem colonization, we used this bacterium to inoculate two legumes which, like *S. rostrata*, are nodulated on both roots and stems, although not by ORS571. These two legumes, *Aeschynomene afraspera* and *A. nilotica*, after inoculation with *Azorhizobium caulinodans* ORS571 (pXLGD4), showed no blue bands in lateral root vascular tissue. However, high magnification light microscopy showed that the azorhizobia had entered some of the cracks formed around emergent lateral roots, and had proceeded to invade adjacent intercellular spaces. We also inoculated tube-grown *S. rostrata* with *Rhizobium meliloti* RCR2011 (pXLGD4), which nodulates alfalfa and, again, blue bands were not found in lateral roots. Moreover, blue precipitate could not be found in lateral root xylem, even when specimens were observed by high magnification light microscopy. These cross-inoculation experiments indicated that ORS571 specifically colonized xylem elements of *S. rostrata*, its own symbiotic partner. *S. rostrata* is also nodulated on roots and stems by *Sinorhizobium teranga* ORS604 and *S. saheli* ORS611. These two sinorhizobia, both modified by introduction of pXLGD4 carrying the *lac*Z gene, were used to inoculate tube-grown plants of *S. rostrata*. Xylem colonization occurred with both ORS604 and ORS611 (table 1).

(*c*) *The role of Nod factors*

We examined whether rhizobial Nod factors, lipo-chito-oligosaccharides produced after translation of

Table 1. *Relationships between nodulation and xylem colonization in* Sesbania rostrata *and* Aeschynomene *species inoculated with various strains of rhizobia*

plant	rhizobia species	rhizobia strain	nodulation and N_2 fixation stems	roots	nif	root xylem colonization
Sesbania rostrata	*A. caulinodans*	ORS571	+	+	+	+[A]
		ORS571(pXLGD4)	+	+	+	+[B]
	R. meliloti	RCR2011(pXLGD4)	−	−	−	−[C]
	S. saheli	ORS611(pXLGD4)	+	+	+	+[B]
	S. teranga	ORS604(pXLGD4)	+	+	+	+[B]
Aeschynomene afraspera, *A. nilotica*	*A. caulinodans*	ORS571(pXLGD4)	−	−	−	−[C]

[A]detected microscopically; [B]detected microscopically and by *lacZ* reporter gene; [C]no xylem colonization, but some colonization of root intercellular spaces.

rhizobial *nodABC* genes (Long 1996), were involved in xylem colonization in *S. rostrata*, and whether xylem colonization was dependent upon or followed nodulation. We found that the mutant, ORS571::*nodC*(pXLGD4), which is deficient in Nod factors and which does not induce nodules in *S. rostrata*, still colonized lateral root xylem in this legume. Approximately 25% of tube-grown plants inoculated with this *nodC* mutant strain showed blue linear bands. Light and electron microscopy revealed bacterial colonization to be similar to colonization by ORS571(pXLGD4). The *nodC* mutant invaded the plant through the annular cracks at the bases of emergent lateral roots by the same route as the wild-type bacterium (Ndoye *et al.* 1994). Bacteria re-isolated from plants of *S. rostrata* inoculated with ORS571::*nodC*(pXLGD4) showed typical azorhizobial colony morphology, did not take up Congo Red dye (normally taken up by bacterial contaminants), exhibited resistance to tetracyclin and kanamycin, and grew as blue colonies on selection medium containing X-Gal.

4. DISCUSSION

This study demonstrates that *Azorhizobium caulinodans* ORS571 is able to colonize the xylem of the roots of *Sesbania rostrata*. We found the *lacZ* assay to be reliable for unambigously locating azorhizobia in xylem; regions of blue precipitate produced by azorhizobia could be distinguished easily from blue-staining plant material. The presence of azorhizobia in the xylem was confirmed by light and electron microscopy. Bacteria re-isolated from inoculated plants and plated onto selective media containing X-Gal, produced blue-staining colonies, confirming that some azorhizobia still retained pXLGD4. Re-isolated bacteria also exhibited typical azorhizobial colony morphology on semi-solidified TY medium and failed to take up Congo Red dye (a diagnostic test for rhizobia; Somasegaran & Hoben 1994). Collectively, these results constitute strong evidence for xylem colonization of *S. rostrata* by ORS571. This information extends our current knowledge of xylem colonization by bacteria, which has already been demonstrated for agrobacteria in *S. rostrata* (Vlachova *et al.* 1987) and for *Acetobacter diazotrophicus* in sugar cane (James *et al.* 1994). In fact, the number of plant species found to exhibit xylem colonization by bacteria without plant disease symptoms has increased dramatically in recent years, suggesting that xylem colonization is a common aspect of plant–microbe interactions (Kloepper *et al.* 1992).

The blue bands produced by ORS571 and *Sinorhizobium* spp. carrying *lacZ* were seen only in lateral roots that had emerged through the primary root epidermis. Generally, the bands were confined to a region of the laterals within, or extending just outside, the primary root, even when laterals were of considerable length. These observations suggest that after entry into natural fissures created during emergence of a lateral root (so-called crack entry, i.e. intercellularly between adjacent cells without the formation of infection threads; Ndoye *et al.* 1994), *Azorhizobium caulinodans* ORS571 sometimes proceeds to colonize xylem elements in the same lateral. This is not surprising, since nodules and lateral roots both form opposite the protoxylem poles of the central stele (Rolfe & Gresshoff 1988). Consequently, lateral root xylem parenchyma tissue lies adjacent to the usual rhizobial invasion route to nodule meristems. Blue precipitate was sometimes observed in primary root xylem, adjacent to lateral root xylem elements containing precipitate (figure 3*b*). It is possible that the transpiration stream may move azorhizobia from the lateral root xylem into that of the primary root.

Our finding that crack-entry and xylem colonization of *S. rostrata* lateral roots by ORS571 is Nod factor independent, is unexpected, and raises questions concerning the mechanisms that control these aspects of the interaction in this legume. It is noteworthy that the colonization of lateral root cracks of several non-legumes by ORS571 is also Nod factor independent, and is also stimulated by flavonoids (Gough *et al.* 1996). There is a need to determine whether crack entry and xylem colonization in *Sesbania rostrata* is also enhanced by flavonoids.

It will be interesting to determine whether ORS571 colonizes the xylem of legumes such as *Leucaena* spp., which produce ineffective nodules after inoculation with this bacterium (Boivin *et al.* 1997). Since most legumes are invaded by rhizobia through root hairs and not by crack entry, xylem colonization may not occur in the majority of legumes. It will therefore be important to assess whether xylem colonization occurs

only in those plant–rhizobia symbioses characterized by crack entry bacterial invasion, as in *Lupinus* and *Arachis* spp., and in the non-legume genus *Parasponia*.

Rhizobia have been described as refined parasites, with xylem-inhabiting endophytes occupying a more primitive phylogenetic position (Djordjevic *et al.* 1987); ORS571 appears to exhibit an interesting mixture of these phenotypic traits. Our four month-old plants of *S. rostrata* inoculated with ORS571 showed no signs of disease. Moreover, xylem elements containing azorhizobia were not occluded by plant-derived materials (figure 2*a*,*b*). The *lacZ* reporter gene, inserted into *Pseudomonas* spp., has been used to examine xylem colonization associated with plant disease in tomato (Vasse *et al.* 1995). Non-rhizobial vascular colonization sometimes leads to plant disease, but it is becoming increasingly evident that healthy plants often contain some benign vascular endophytes (Bell *et al.* 1995), without impairment of transpiration (van Alfen 1982). Moreover, xylem-inhabiting bacteria can sometimes benefit the plant by enhancing shoot growth (Kloepper *et al.* 1992). Our finding that rhizobia can colonize, concomitantly, both xylem and nodules, provides the novel perspective that some rhizobia can exist as symbionts in nodules and as benign vascular endophytes. Alternatively, it is possible that the vascular rhizobial endophytes could also be symbiotic, i.e. contribute fixed nitrogen to their host plant.

There is evidence that at least part of the mechanism necessary for nodule formation in legumes is also present in non-legumes (Röhrig *et al.* 1995). Even without nodule formation, colonization of the xylem of sugar cane by diazotrophs has been shown to provide substantial amounts of fixed nitrogen to this non-legume (Boddey *et al.* 1995). Recently, endophytic colonization of the vascular tissue of sorghum leaves (by a species of nitrogen-fixing *Herbaspirillum*) has been demonstrated (James *et al.* 1997). Therefore, we are currently evaluating whether xylem colonization in *S. rostrata* contributes to nitrogen fixation, using ORS571::*nodC*(pXLGD4) which, as we have shown, colonizes the xylem but does not induce nodules in *S. rostrata*. The results of our host range experiments using *Aeschynomene* spp. inoculated with ORS571, and *Sesbania rostrata* inoculated with ORS571, ORS604 or ORS611, show that lateral root xylem elements of *S. rostrata* are colonized after invasion from the rhizoplane, but only by the natural symbiont that also nodulates the plant. However, xylem colonization has been observed in wheat which does not develop nodules; plants were pot-grown and inoculated repeatedly with *Azorhizobium caulinodans* (Sabry *et al.* 1997). Therefore, under some growth conditions, *A. caulinodans* ORS571 may be able to colonize the xylem of plants other than *S. rostrata*.

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