Endophytic establishment of *Azorhizobium caulinodans* in wheat

SAMI R. S. SABRY[1,2], SALEH A. SALEH[1,3], CAROLINE A. BATCHELOR[1], JUNE JONES[1], JOAN JOTHAM[1], GORDON WEBSTER[1], SHANKER L. KOTHARI[1,4], MICHAEL R. DAVEY[1] AND EDWARD C. COCKING[1]

[1] *Plant Genetic Manipulation Group, Department of Life Science, University of Nottingham, Nottingham NG7 2RD, UK*
[2] *Wheat Research Department, Field Crops Research Institute, Agricultural Research Center, Giza 12619, Egypt*
[3] *Agricultural Microbiology Research Section, Soils and Water Research Institute, Agricultural Research Center, Giza 12619, Egypt*
[4] *Department of Botany, University of Rajasthan, Jaipur-302004, India*

SUMMARY

Nitrogen fixing nodules are formed on the roots and stems of the tropical legume *Sesbania rostrata* by *Azorhizobium caulinodans* as a result of crack entry invasion of emerging lateral roots. Advantage was taken of this invasion capability of *A. caulinodans* to determine whether inoculation of the non-legume wheat with *A. caulinodans* would result in the endophytic establishment of azorhizobia within wheat roots. Advantage was also taken of the oxygen tolerance of the nitrogenase of free-living azorhizobia to assess the extent to which the endophytic establishment of azorhizobia in wheat roots would provide a niche for nitrogen fixation of benefit to the plant. Wheat was inoculated with *A. caulinodans* and grown in pots under controlled conditions, without added growth regulators and without addition of fixed nitrogen. Microscopic examination of the short lateral roots of inoculated wheat showed invasion of azorhizobia between cells of the cortex, within the xylem and the root meristem. Acetylene reduction assays combined with analysis of tissue nitrogen levels indicated the likelihood that colonization led to nitrogenase activity. Inoculated wheat showed significant increases in dry weight and nitrogen content as compared with uninoculated controls. We discuss the extent to which this nitrogen fixation is likely to involve symbiotic nitrogen fixation, and we indicate the need for field trials to determine the extent to which inoculation of wheat with *A. caulinodans* will reduce the requirement for inputs of nitrogenous fertilizers.

1. INTRODUCTION

We have investigated whether *Azorhizobium caulinodans* ORS571 (Dreyfus *et al*. 1988), which is known to form both root and stem nodules on the tropical legume *Sesbania rostrata* as a result of crack entry invasion of emerging lateral roots (Tsien *et al* 1983; Ndoye *et al*. 1994), might also invade a non-legume crop such as wheat by crack entry. The establishment of *A. caulinodans* in the root system of wheat without nodule formation would be a novel situation in this non-legume crop.

Previous attempts to establish an endophytic nitrogen fixing interaction of *A. caulinodans* with wheat have utilized the addition of plant growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D) to induce nodular structures. However, only low levels of nitrogenase activity were observed, and the addition of 2,4-D was generally detrimental to plant growth (Chen *et al*. 1992; Yu & Kennedy 1995). Somewhat similar results have also been reported for rice inoculated with azorhizobia (Christiansen-Weniger 1996). Nitrogenase activity was not detected in studies of the interaction between rhizobia and cultured potato tissues (Spencer *et al*. 1994). Our previous experiments with wheat avoided the addition of plant growth regulators and utilized seedlings grown aseptically in tubes for a few weeks under controlled conditions and inoculated with *A. caulinodans*. Although nodular structures were not formed, evidence was obtained for the crack entry invasion of emerging lateral roots by *A. caulinodans*, initially at low frequency (Cocking *et al*. 1992, 1994) and, recently, at higher frequency with stimulation by specific flavonoids (Gough *et al*. 1996). Low levels of nitrogen fixation were recorded using the acetylene reduction assay (Cocking *et al*. 1995). Quispel (1991) has suggested that only in endophytic systems are the prerequisites for effective nitrogen fixation likely to be fulfilled in non-legume rhizobial interactions.

In this work we have extended our earlier studies with wheat in tubes (Cocking *et al*. 1995; Gough *et al*. 1996) by inoculating wheat with *A. caulinodans* and growing the plants under controlled conditions in pots for up to eight weeks. Detection of significant levels of nitrogenase activity in these inoculated plants, grown without the addition of plant growth regulators, led us to investigate whether nitrogen fixation resulting from a more extensive endophytic establishment of *A.*

caulinodans in the wheat root system was resulting in significant increases in plant nitrogen and dry weight. We critically discuss the extent to which the endophytic establishment of nitrogen fixing *A. caulinodans* that we have observed, and the resultant increases in nitrogen content and dry weight, might involve endosymbiotic nitrogen fixation.

2. MATERIALS AND METHODS

(*a*) *Cultures of rhizobia*

Azorhizobium caulinodans ORS571 (IRBG314), *A. caulinodans* ORS571 (Gent), *A. caulinodans* ORS571 (pGV910-C1) and *Bradyrhizobium* spp. ORS310 were supplied by J. K. Ladha, IRRI (IRBG314), D. Geelen, Gent (ORS571, Gent and pGV910-C1) and D. Alazard, Senegal (ORS310), respectively. *A. caulinodans* ORS571 (pGV910-C1) was an enodoglucanase mutant (Geelen *et al*. 1995). *Bradyrhizobium* spp. ORS310, with a nitrogenase tolerance of up to 0.5 % oxygen in the gas phase (Alazard 1990), was originally isolated from stem nodules of *Aeschynomene indica*. The three *Azorhizobium* strains were grown in TGYE medium (Ladha *et al*. 1989), while *Bradyrhizobium* spp. ORS310 was cultured in YEM medium (Vincent 1970). Nif$^-$ *A. caulinodans* (ORS571, 57022, *nif22*-Tn5) supplied by C. Elmerich, Paris, were grown in LSO medium (Denèfle *et al*. 1987). By using the *nif* H gene from *Rhizobium phaseoli* strain CFN-42 as a probe, all the *A. caulinodans* strains were shown to belong to the same bacterial group; ORS310 belonged to a separate group (R. Palacios, personal communication).

(*b*) *Plant material*

The breadmaking wheat (*Triticum aestivum* L.) cultivar Canon was supplied by Plant Breeding International, Cambridge, UK and the cultivar Giza 164 by the Agricultural Research Centre, Giza, Egypt. Seeds were surface-sterilized using 30 % (v/v) Domestos bleach (Lever Industrial, Runcorn, UK) for 60 min and washed five times in sterile distilled water before planting (two seeds/sterilized pot) in a vermiculite and perlite (1:1, v:v) mixture (200 g/pot). After emergence, seedlings were thinned to one per pot. They were watered daily with nitrogen-free nutrient solution (Fähraeus 1957). Plants were maintained in a growth chamber (S.B. Refrigeration, Nottingham) with a 14 h photoperiod (250 μM s^{-1} m^{-2} white fluorescent illumination), day and night temperature maximum and minimum of 24 °C and 20 °C respectively, and 70 % relative humidity; aseptic precautions were taken as fully as possible. *Sesbania rostrata* seeds, supplied by J. K. Ladha, IRRI, were scarified in concentrated sulphuric acid for 20 min, sown in a vermiculite–perlite mixture and grown under the same conditions as wheat.

(*c*) *Inoculation of wheat and* Sesbania rostrata

Because plants were watered daily, 2 ml of rhizobial culture (approx. 10^9 bacteria/ml) were added four times to each wheat plant, once at planting of the seeds, and subsequently three times at one-week intervals. *Sesbania rostrata* seeds and plants were inoculated similarly. Uninoculated wheat plants received only the bacterial culture medium. For nitrogen fertilizer treatments of uninoculated wheat, a total of 60 mg of ammonium nitrate was added to each pot in three doses; 30 mg, 15 mg and 15 mg at one, two and three weeks after planting, respectively.

(*d*) *Dry weight and total nitrogen determinations*

Plant dry weight was determined by drying shoots at 70 °C to constant weight. Near infrared reflectance spectroscopy calibrated against the N content (Kjeldahl) of dried wheat shoots was used to determine the total N content (Batten *et al*. 1991).

(*e*) *Nitrogenase activity determinations*

For each acetylene reduction assay of nitrogenase activity, five plants were removed from their pots, shaken gently to remove the vermiculite–perlite mixture and each incubated with 10 % (v/v) acetylene and 90 % air at room temperature in a closed system (500 ml) (Turner & Gibson 1980). Samples were assayed for ethylene production using a Pye Unicam PU4500 gas chromatograph. There was a lag phase of a few hours followed by an approximately linear production of ethylene.

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

Roots were fixed in 2.0 % (v/v) glutaraldehyde for 24 h at 4 °C, followed by 1.0 % (w/v) osmium tetroxide (2 h, 4 °C). Fixatives were prepared in 0.1 M sodium phosphate buffer, pH 7.0. Specimens were dehydrated through 10 % (v/v) increments of ethanol to absolute ethanol (30 min each) and embedded in LR White medium grade resin (The London Resin Co., Basingstoke, UK) (Davey *et al*. 1993). For light microscopy, sections were cut to 2 μm and stained with 0.5 % (w/v) toluidine blue in 0.1 % (w/v) sodium tetraborate (2 min, 60 °C). Ultra-thin sections were stained with lead citrate and examined at 80 kV in a Jeol 100-S transmission electron microscope (Davey *et al*. 1993).

3. RESULTS

(*a*) *Nitrogenase activity of wheat inoculated with rhizobia*

Two cultivars of wheat, namely Canon (Spring wheat) and Giza 164, of different genetic backgrounds widely grown in the UK and Egypt respectively, were selected for inoculation with rhizobia and assayed for nitrogenase activity using the acetylene reduction assay. Comparative assessments were made of nitrogenase activity at five and eight weeks after inoculation with *A. caulinodans* ORS571 (IRBG314 and Gent), *A. caulinodans* ORS571 (pGV910-C1) and *Bradyrhizobium* spp. ORS310 (table 1). At five weeks post-inoculation, pot grown wheat plants (cultivar Canon) inoculated with *A. caulinodans* IRBG314 showed a high level of ethylene production in the acetylene reduction assay, which was maintained at eight weeks; plants inoculated with the other bacterial strains showed little or no activity at five weeks, but this increased significantly at eight weeks. The cultivar Giza 164 exhibited a delayed response compared with the cultivar Canon when inoculated with *A. caulinodans* IRBG314 and with the other rhizobia, but showed a high level of ethylene production in the acetylene reduction assay with all inocula at eight weeks. Reduction of acetylene to ethylene was not detected when plants of Canon and Giza 164 were uninoculated, or inoculated with the

Table 1. *Nitrogenase activity of wheat inoculated with rhizobia*

(Ethylene was not produced in the acetylene reduction assay when the wheat varieties were uninoculated, or inoculated with a Nif$^-$ strain of *A. caulinodans*. Means were obtained from five plants ± standard deviation.)

		nmoles ethylene/plant/24 h	
wheat cultivar	rhizobia	5 weeks	8 weeks
Canon	*A. caulinodans* (IRBG314)	1435 ± 639	1477 ± 359
	A. caulinodans (Gent)	0	1140 ± 1211
	A. caulinodans (pGV910-C1)	151 ± 106	429 ± 201
	Bradyrhizobium ORS310	0	269 ± 235
Giza 164	*A. caulinodans* (IRBG314)	221 ± 174	1150 ± 1010
	A. caulinodans (Gent)	0	1206 ± 625
	A. caulinodans (pGV910-C1)	0	1512 ± 1224
	Bradyrhizobium ORS310	0	1021 ± 737

Table 2. *Dry weight and nitrogen content of wheat after eight weeks*

(Comparison between the treatment means for dry weight (mg plant^{-1}) and nitrogen content (mg plant^{-1}) using the *t*-test for the difference between the uninoculated treatment and the other five treatments. Treatment means were obtained from five plants.)

				inoculated			
wheat cultivar	plant shoot	uninoculated	uninoculated plus NH_4NO_3	ORS571 (IRBG314)	ORS571 (Gent)	ORS571 (pGV910-C1)	ORS310
Canon	dry wt (mg)	220	631**	671**	383**	528**	333
	N content (mg)	3.02	13.25**	8.92**	7.85**	8.55**	4.03**
Giza 164	dry wt (mg)	253	628**	556**	366*	508**	514**
	N content (mg)	3.11	8.34**	6.33**	6.84**	7.41**	5.80**

* Significant at 0.05 level of probability.
** Significant at 0.01 level of probability.

Nif$^-$ strain of *A. caulinodans*. There was no endogenous ethylene production when samples were assayed without acetylene.

(*b*) *Comparison of the nitrogenase activity of wheat with* Sesbania rostrata *inoculated with* A. caulinodans

The nitrogenase activity of *Sesbania rostrata* plants inoculated with *A. caulinodans* IRBG314 was compared with wheat plants (cultivar Canon) also inoculated with *A. caulinodans* IRBG314 under similar growth and acetylene reduction assay conditions. For inoculated plants of the legume *Sesbania rostrata*, nodulated on both stem and roots, the mean activity for five plants at five and at eight weeks was 10 104 ± 9086 and 11 879 ± 7263 nmoles ethylene/plant/24 h respectively. A comparison with wheat, inoculated with *A. caulinodans* IRBG314 (table 1), showed that the mean activity of inoculated wheat was approximately one-eighth the mean activity of *S. rostrata* inoculated under similar conditions.

(*c*) *Dry weight and nitrogen content of inoculated and uninoculated wheat*

As the reduction of acetylene to ethylene procedure provided only an approximate estimate of nitrogenase activity at the time of the assay, measurements were taken of the dry weight and nitrogen content of the uninoculated and inoculated wheat varieties at eight weeks (table 2). These assessments provided an integrated measurement of the consequences of nitrogenase activity detected at five weeks and eight weeks, as summarized in table 1. A significant increase was observed in the dry weight and nitrogen content of the shoot systems of all inoculated wheat plants compared with uninoculated plants. In table 2, comparisons were also made at eight weeks of the dry weight and nitrogen content of the two uninoculated wheat varieties with that of the two wheat cultivars uninoculated, but supplied with NH_4NO_3 (uninoculated plus NH_4NO_3 column, table 2), equivalent to fertilizer application in the field of 240 kg N ha^{-1}.

(*d*) *Effects of inoculation on wheat roots*

The root systems of the inoculated wheat plants at five and eight weeks showed, in comparison with the uninoculated controls, a general stimulation of root development and the formation of large numbers of short lateral roots. Five weeks after inoculation with *A. caulinodans* IRBG314, there were approximately five times more short lateral roots, each up to 3 mm in length, present on inoculated wheat as compared with the root system of uninoculated plants. Light

microscopy of sections of a random sample of 50 of these short lateral roots, from wheat plants five weeks after inoculation, showed that approximately half of the short lateral roots were being invaded by bacteria, presumed to be azorhizobia. The invading bacteria could be seen within cells of the meristems of the short lateral roots (figure 1*a*), between the cells of the cortex (figure 1*b*) and within some of the xylem elements (figure 1*c*). No bacteria were observed in uninoculated wheat plants similarly analysed.

4. DISCUSSION

It has been suggested that the special invasive properties of *A. caulinodans* ORS571 and *Bradyrhizobium* spp. ORS310, are probably associated with their secretion of cellulases and pectinases, which enable these bacteria to penetrate between the cells of the emerging young lateral roots (Cocking *et al*. 1994). The fact that enhancement of cellulase activity in *A. caulinodans* (pGV910-C1) did not significantly increase nitrogenase activity of inoculated wheat suggests that cellulase activity in the wild-type strain is adequate for infection.

The interaction of azorhizobia with wheat roots exhibits similarities to the invasion of the xylem vessels of roots of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus* (James *et al*. 1994), and by *Herbaspirillum* spp. (Döbereiner *et al*. 1995), and of wheat by *Pantoea agglomerans* (Ruppel *et al*. 1992). It was suggested that xylem vessels are possible sites of nitrogen fixation by diazotrophs, as the xylem elements could provide the low pO_2 and a source of carbohydrate for nitrogenase activity. A comparable low pO_2 may also occur in the xylem of wheat inoculated with azorhizobia. In tube experiments, we have detected significant crack entry colonization of the lateral root bases of wheat by ORS571 carrying a constitutive *lac*Z reporter gene (Gough *et al*. 1996). There may also be similarities between the crack entry invasion of wheat lateral roots by these stem-nodulating rhizobia and the very occasionally observed penetration of rhizobia into lateral roots of some legumes. In alfalfa and lupin, it was observed early this century (Moore 1905) that it was possible for rhizobia to penetrate small roots and to be of benefit to the plant, without the formation of nodules on the host or any external evidence of their presence.

The acetylene reduction assay provides only an approximate measure of nitrogenase activity (Boddey 1987), and it will be necessary to confirm the acetylene reduction assay results using $^{15}N_2$. The significant increase in nitrogenase activity that we have observed in inoculated pot-grown wheat plants suggests that azorhizobia are more strongly induced to establish themselves endophytically under these pot conditions than under restricted growth conditions in tubes. We were able to demonstrate, in tube experiments, that conditions were appropriate for nitrogen fixation at colonization sites in wheat by detecting expression of a *nif* promoter-*lac*Z gene. However, we could only detect nitrogenase activity by the acetylene reduction assay when succinate was added to the plant growth medium, indicating that, in tubes, carbon is limiting for nitrogen fixation (Gough *et al*. 1996). It would seem from the present results that an adequate supply of carbon is being provided from photosynthesis in pot-grown wheat plants. The observed significant increases in plant dry weight and nitrogen content of both the inoculated wheat varieties, as compared with uninoculated wheat, with extensive invasion of their lateral roots, indicates that lateral roots invaded by azorhizobia are providing a niche for nitrogen fixation of some benefit to the plant. The beneficial effect of inoculation with azorhizobia on dry weight and nitrogen content can sometimes equate to the beneficial effects of substantial additions of nitrogenous fertilizer to uninoculated controls. In all experiments, the uninoculated controls had the same low input of fixed nitrogen from the vermiculite, perlite and growth media as the inoculated wheat. Other diazotrophs have been observed to behave differently. In wheat inoculated with *Azospirillum*, even though a somewhat comparable stimulation of root development and lateral formation has been observed (Bothe *et al*. 1992), no significant contribution has generally been found from biologically fixed nitrogen to the nitrogen demand of the plant (Michiels *et al*. 1989). Also, even when extensive invasion occurs, such as in the interaction of Kallar grass and rice with *Azoarcus*, increases in growth and protein content may not be as a direct result of nitrogen fixation (Hurek *et al*. 1994).

The assessment of the extent to which there is an actual symbiotic fixation of nitrogen is made difficult by the known ability of *A. caulinodans* to fix nitrogen asymbiotically at up to 3% O_2 in the free-living state (Dreyfus *et al*. 1983) as well as symbiotically in nodules, and by the failure of free-living *A. caulinodans* to release the nitrogen that it fixes (Gebhardt *et al*. 1984). Only when invasion of the xylem elements and meristematic cells by azorhizobia occurs may the pO_2 be low enough for nitrogenase activity (Bergersen *et al*. 1988) and for the release of fixed nitrogen from the azorhizobia. Also, although the use of $^{15}N_2$ (Boddey 1987) could assist in establishing that there is incorporation of fixed nitrogen into wheat plants, there would still be the possibility that azorhizobia on the surface of wheat roots, and not bacteria either within or between cells, contribute significantly to the nitrogen fixation observed. In our acetylene reduction assays the presence of approximately 18% O_2 is likely to inhibit the nitrogenase activity of free-living surface azorhizobia (Kennedy & Tchan 1992). We have also attempted to minimize any extraneous microbial contamination by surface sterilizing the wheat seeds and by maintaining aseptic conditions as far as possible in the growth rooms.

It is yet to be determined whether this endophytic establishment of *A. caulinodans* in wheat without nodulation, with its demonstrated ability to decrease the need for fixed nitrogen inputs under our carefully controlled inoculation and growth conditions, will help significantly in agricultural practice. Field trials are now being undertaken, employing a range of wheat varieties and inoculation protocols with *A. caulinodans*, to quantify increases in the availability of biologically

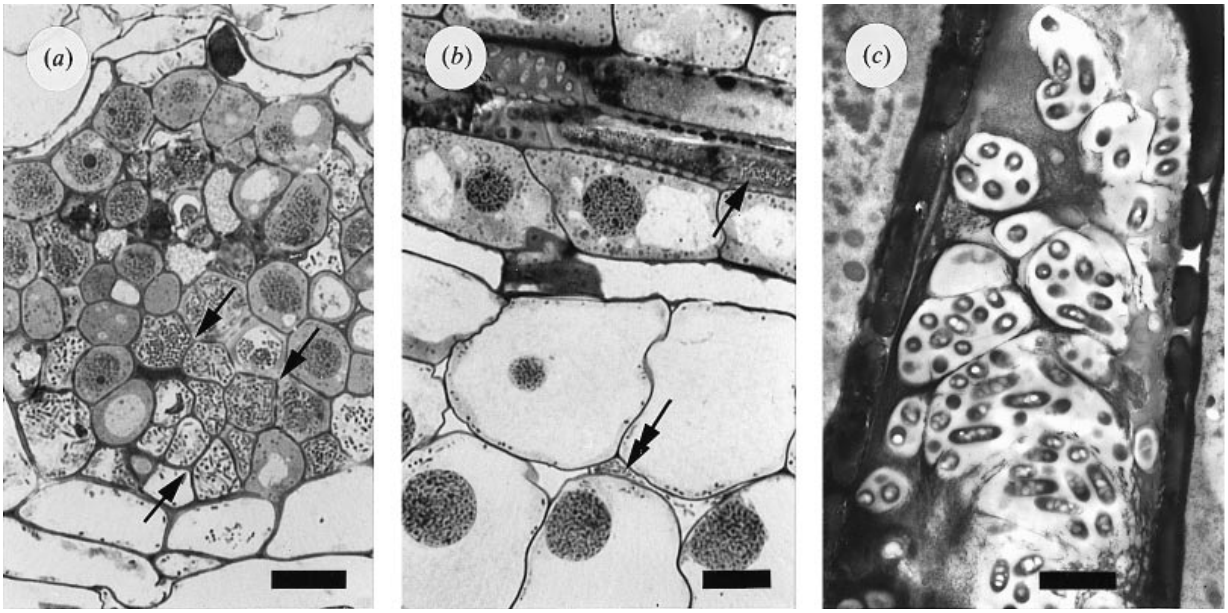

Figure 1. Micrographs of sections of short lateral roots of five-week-old wheat plants invaded by azorhizobia. (*a*) Light micrograph of a root meristem cut obliquely with cells containing azorhizobia (arrowed). Bar, 20 μm. (*b*) Azorhizobia between cells of the cortex (double arrow) and within a xylem element (single arrow). Bar, 20 μm. (*c*) Electron micrograph showing azorhizobia, surrounded by fibrillar material, within a xylem element. Bar, 1.8 μm.

fixed nitrogen, and the extent to which nitrogenous fertilizer inputs can be reduced without yield losses.

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