Planta (1990) 180:303–311

Planta
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Interaction of *Rhizobium fredii* USDA257 and nodulation mutants derived from it with the agronomically improved soybean cultivar McCall

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Abstract. *Rhizobium fredii* USDA257 does not nodulate McCall soybean (*Glycine max* (L.) Merr.), but two transposon-mutants derived from it, 257DH4 and 257DH5, do. All three organisms cause curling of McCall root hairs and induce the formation of underlying cortical cell divisions. The mutants produce infection threads, and many of the meristematic foci develop into nodules. In contrast, root hairs that deform in response to USDA257 lack infection threads, and meristematic activity ceases prior to the appearance of nodule meristems. Root systems nodulated by mutant 257DH4 reduce acetylene at rates similar to those of roots nodulated by reference *R. fredii* strain USDA191. The presence of living cells of USDA257 in inocula leads to strong inhibition of nodulation by 257DH4 but not by 257DH5. This blocking effect depends on the ratio of USDA257 cells to 257DH4 cells in the inoculum; nodules that form contain cells of 257DH4, but not those of parental strain USDA257.

Key words: *Glycine* (nodulation) – Infection threads – Nodulation, competitive blocking – Nodulation mutants – *Rhizobium*

Introduction

Both *Bradyrhizobium japonicum* and *Rhizobium fredii* produce nitrogen-fixing nodules on the roots of soybean, *Glycine max* (L.) Merr. Bradyrhizobia are the classical symbionts of this agriculturally important legume, but they have certain drawbacks as research tools: Their growth rates in culture can be exceedingly slow, and their *nod* genes are chromosomal and hence relatively inaccessible. *Rhizobium fredii* was first described in the early 1980's (Keyser et al. 1982; Scholla and Elkan 1984), and it differs markedly from *B. japonicum*. It is a fast-growing species, and like most other rhizobia, its *nod* genes are borne on large sym plasmids (Appelbaum et al. 1985a). As a consequence, *R. fredii* is receiving increasing attention from those seeking to understand the genetics of nitrogen-fixing symbioses with soybean.

The ability of *R. fredii* to nodulate and fix nitrogen in association with soybean is strain-dependent and quite variable. Some strains, exemplified by USDA191, are effective with both primitive cultivars that have never been subjected to agronomic improvement, and with advanced cultivars that have been released by plant breeders (Scholla and Elkan 1984; Dowdle and Bohlool 1985; Lin et al. 1987). Other strains of *R. fredii*, including 10 of the 11 that were first described, are effective only with unimproved cultivars such as Peking (Keyser et al. 1982). We have been interested in USDA257, a strain of the second type, which effectively nodulates primitive soybean genotypes, but fails to nodulate the improved cultivar McCall (Heron and Pueppke 1984). Using transposon Tn*5* mutagenesis, we identified five unusual mutants that are derived from USDA257. Each has acquired the ability to nodulate McCall while retaining the other nodulating characteristics of the parent (Heron et al. 1989). Symbiotic characterization of the two genetically best defined mutants is the subject of the research reported here. Mutant 257DH4 contains a single copy of the transposon in the approx. 300 megadalton (MDa) sym plasmid and forms effective nodules on McCall. In contrast, one copy of the transposon is in the chromosome of mutant 257DH5, and 257DH5 nodules on McCall fail to fix nitrogen (Heron et al. 1989).

Similar mutants with an extended host-range have been described in *R. leguminosarum* bv. *trifolii* and *R. meliloti* (Debellé et al. 1986; Horvath et al. 1986; Faucher et al. 1988). The mutations map to the *nod-FEGH* region of the sym plasmid in both organisms and confer the capacity to nodulate pea (*Pisum sativum*)

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or vetch (*Vicia sativa*). These species normally are nodulated by *R. leguminosarum* bv. *viciae*, and they remain uninfected upon inoculation with wild-type *R. leguminosarum* bv. *trifolii* or *R. meliloti*. The extended-host-range mutants of these two species differ from those of *R. fredii* in two fundamental aspects: (i) they are impaired in their capabilities to nodulate their normal hosts, and (ii) their host range is extended to new legume species, and not to new cultivars of the normal host. The mutants of *R. fredii* USDA257 thus offer unique opportunities to examine the basis for host range in legume-*Rhizobium* interactions.

We show here that *R. fredii* USDA257 can curl McCall root hairs, but induces only a few rounds of cell division in the underlying cortical cells and forms no infection threads. The mutants also elicit these host responses, but infection threads ensue, and nodules eventually appear. We also demonstrate that relatively high concentrations of 257DH4 cells are required for optimum nodulation, that acetylene reduction rates of 257DH4 nodules are equivalent to those produced by USDA191, and that nodulation by this mutant is acutely sensitive to the presence of living cells of the parental strain.

Material and methods

Maintenance and growth of organisms. Wild-type *Rhizobium fredii* strains USDA191 and USDA257 were from H.H. Keyser, U.S. Department of Agriculture, Beltsville, Md., USA. The origin of mutants 257DH4 and 257DH5 has been described by Heron et al. (1989). Stocks of the bacteria were stored at $-70°$ C in 7.5% glycerol, and short-term cultures were maintained as slants on yeast extract-mannitol (YEM) medium (Vincent 1970) at 8° C. Rhizobia were routinely grown in liquid YEM medium at 28–30° C and 125 rpm. Cultures of the mutants were supplemented with kanamycin at 100 μg/ml.

Seeds of soybean (*Glycine max* (L.) Merr. cv. McCall) were from D.A. Whited, North Dakota State University, Fargo, USA. They were surface-sterilized and germinated on water agar as described by Pueppke (1983).

Acetylene-reduction assays. Logarithmic-phase bacterial cultures were centrifuged at $7700 \cdot g$ for 10 min and the pellets suspended in sterile distilled water. Cell concentration was adjusted turbidimetrically to $5 \cdot 10^8$/ml. Roots of pregerminated seedlings were dipped into bacterial suspensions and the seedlings immediately transferred to autoclaved plastic growth pouches (Northrup King Co., Minneapolis, Minn., USA). Each pouch contained three seedlings and 15 ml of autoclaved Jensen's N-free solution (Vincent 1970). The pouches were incubated in a growth chamber at 400 μmol photons$\cdot m^{-2} \cdot s^{-1}$ with a 12-h light period daily. Light was from cool white fluorescent lamps (general Electric, Cleveland, Oh., USA). Nodulated root systems were harvested 12, 15, 18, and 21 d after inoculation and assessed for acetylene-reduction activity by the method of Schwinghamer et al. (1970). Two independent experiments were done.

Ultrastructural studies. For light microscopy, seedlings were inoculated with bacteria suspended in Jensen's solution as described above, and the position of each primary root tip was marked on the surfaces of the growth pouches. On the fifth and eighth days after inoculation, a segment of primary root extending from 1 mm above to 9 mm below the root-tip mark was excised from each plant and immersed in ice-cold 100 mM sodium-cacodylate buffer, pH 7.0, containing 2% glutaraldehyde. The root segments were processed and embedded in Paraplast Plus (Fisher Scientific, St. Louis, Mo., USA) as described by Pueppke and Payne (1987). Serial, 15-μm longitudinal sections were cut, stained with safranin-fast green, and examined at magnifications ranging from ×40 to ×400 with a Leitz (Wetzlar, W. Germany) dialux microscope. A total of 44 root segments, from two experiments, were serially sectioned and examined by this method.

Samples for electron microscopy were obtained from seedlings inoculated and marked as described above. Strips of surface tissues within the zone extending from 1 mm above to 9 mm below the root tip mark were harvested 4–5 d after inoculation, and pieces of nodules from the same regions of roots of other plants were harvested 8–9 d after inoculation. Tissues were immediately fixed in 50 mM sodium-cacodylate buffer, pH 7.3, containing 2.5% glutaraldehyde. Nodule samples for transmission electron microscopy (TEM) were further sliced into pieces of approx. 1 mm^3 after fixation for 1–2 h. After fixation for an additional 2 h, all samples were washed with 50 mM sodium-cacodylate buffer, pH 7.3, and post-fixed overnight with 1% osmium tetroxide in the same buffer. The samples then were dehydrated in an ascending ethanol series.

Tissues for TEM were embedded in Spurr's (1969) resin. Thin sections were obtained with a diamond knife and stained with 5% uranyl acetate and triple lead (Sato 1967). Stained sections were examined with a JOEL (Tokyo, Japan) JEM 100B microscope at 100 kV. Tissues for scanning electron microscopy (SEM) were critical-point-dried, sputter-coated with gold, and mounted on stubs for viewing with a JOEL SSM35 microscope operating at 10–20 kV.

Nodulation blocking. Cells of strain USDA257 and mutant 257DH4 were harvested from liquid cultures as described above and mixed in sterile phosphate-buffered saline (PBS: 7.2 g NaCl, 2.79 g Na_2HPO_4, 0.43 g KH_2PO_4/l deionized water, pH 7.2) so that the concentration of mutant 257DH4 was constant at 10^8 cells/ml and that of strain USDA257 varied from 10^7 to 10^9 cells/ml. Sets of pregerminated seedlings were transferred to growth pouches and individually inoculated with 0.25-ml aliquots of bacterial suspension delivered from a micropipette. The position of the primary root tip of each seedling then was marked on the pouches. Roots of control seedlings were dipped into PBS or into PBS containing only mutant 257DH4 (10^8 cells/ml). In some experiments, cells of mutant 257DH5 were substituted for those of 257DH4. In others, suspensions of USDA257 were boiled for 20 min and cooled prior to preparation of the mixtures. Nodules were counted on the eighth day after inoculation and on alternate days thereafter until day 20.

Results

Cellular responses of McCall roots to inoculation. Figure 1 illustrates the responses of McCall root hairs to *R. fredii.* All samples were obtained 4–5 d after inoculation, from portions of primary roots known to have been maximally susceptible to nodulation at the time of inoculation (Calvert et al. 1984; Pueppke and Payne 1987). As expected, control root hairs remained elongate and showed no signs of deformation that could be associated with rhizobia (Fig. 1A). Roots inoculated with nodulating reference strain USDA191, however, contained numerous patches of truncated and deformed root hairs, many of which had assumed the shapes of question marks (Fig. 1C gives a representative view).

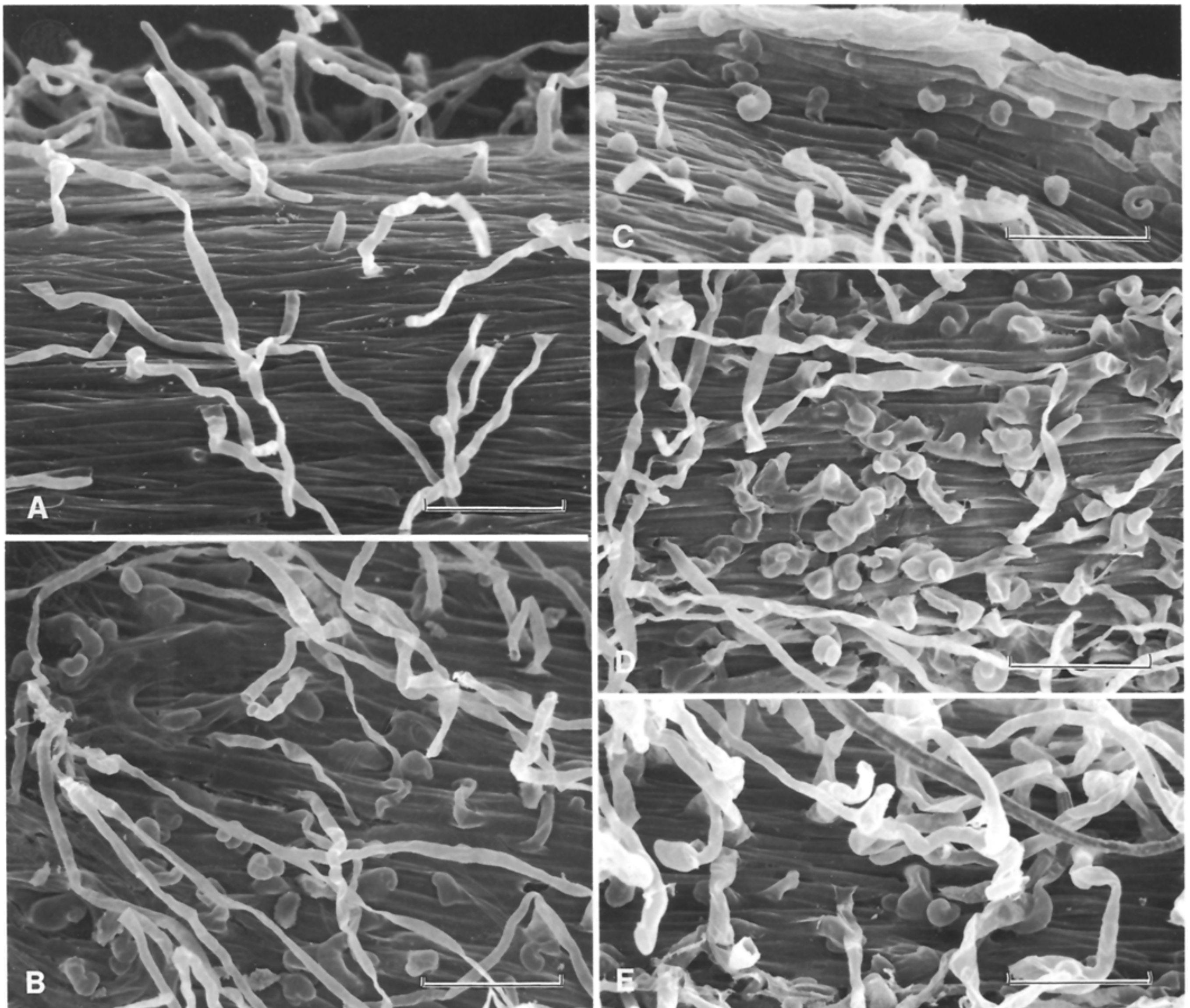

Fig. 1 A–E. Scanning electron microscopy of root hairs on McCall soybeans inoculated with different strains of *Rhizobium fredii*. Seedlings were dipped into bacterial suspensions containing 10^9 cells/ml and transferred to plastic growth pouches. Prescribed zones of the primary roots were sampled on the fourth and fifth days after inoculation as described in *Material and methods*. **A** Surface of a control root treated with N-free solution. Views of roots inoculated **B** with parental strain USDA257, **C** nodulating reference strain USDA191, **D** mutant 257DH4, and **E** mutant 257DH5. Note that in each case, inoculation causes a subpopulation of root hairs to remain short and curl or otherwise deform. Bars = 100 μm, × 200

The root hairs surrounding these patches were indistinguishable from those shown in Fig. 1 A. Figure 1 B, D–E shows that non-nodulating strain USDA257 and both mutants also induce areas of compact, deformed root hairs on McCall soybean. Although the morphologies of root hairs deformed by the four organisms were not noticeably different, zones of responding root hairs were much less frequent on plants inoculated with USDA257 (Fig. 1 B was selected to illustrate one of the rare patches of deformation in response to USDA257).

Development of cortical cell divisions, infection threads, and nodule meristems in response to nodulating reference strain USDA191 was essentially the same as that described by Calvert et al. (1984) for *B. japonicum*. Responses of underlying cortical tissues to inoculation with USDA257 and the mutants are shown in Fig. 2. Although the parental strain and both mutants each induced foci of dividing cortical cells in McCall soybean (Fig. 2 A–C), the developmental patterns were distinct. As is the case with soybean inoculated with *B. japonicum* (Calvert et al. 1984), foci in roots inoculated with USDA257 often were centered beneath curled root hairs (Fig. 2 A). Cell-wall thickenings within individual curled root hairs sometimes were apparent, but infection threads were never observed. Five days after inoculation, all foci were of Types I–III, as defined by Calvert et al.

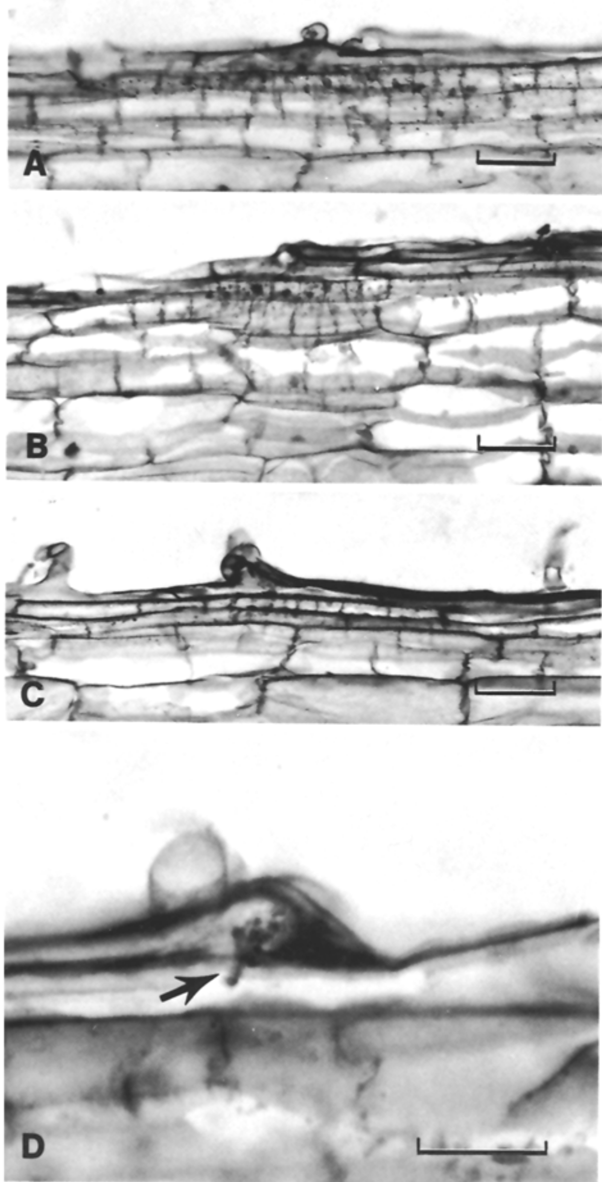

Fig. 2A–D. Meristematic responses of McCall soybean roots to *R. fredii*. Seedlings were dipped into bacterial suspensions containing 10^9 cells/ml and transferred to plastic growth pouches as described in *Material and methods*. Samples were harvested on the fifth and eighth days after inoculation. Bars = 10 μm. The treatments were as follows: **A** Parental strain USDA257, ×200; **B** mutant 257DH4, ×200; **C** mutant 257DH5, ×200. **D** is a higher-magnification view of an infection thread (*arrowhead*) produced by mutant 257DH5 in a curled root hair, ×340

(1984), i.e. cell divisions generally were restricted to the outer cortex, and nodule meristems were not formed. Foci had not developed further in root segments harvested 8 d after inoculation.

Roots inoculated with the mutants produced both infection foci and infection threads (Fig. 2B–D). At 5 d after inoculation, roots that had been inoculated with 257DH4 contained an average of six infection threads per 1-cm segment. Most foci were restricted to the outer cortex, but a few had organized nodule meristems. Small nodules protruded from such roots by 8 d after inoculation. In contrast, 1-cm segments of roots that had been exposed to 257DH5 each had on average less than one infection thread at 5 d after inoculation. With one or two exceptions, infection foci had not enlarged beyond the outer cortex. By day 8, infection threads (Fig. 2D) averaged two per segment, but foci still were of Types I–III. Foci were absent from uninoculated roots.

Infection threads in young nodules produced by reference strain USDA191 are shown in Fig. 3. Threads were associated with cell walls and surround masses of bacterial cells lying side by side. Although bacteroids have not yet been released, the host cells contain dense cytoplasm and have only small vacuoles. Figure 3B confirms that infection threads produced by mutant 257DH4 in curled root hairs function to convey the bacteria into the developing nodule. A comparison of Fig. 3A and B also shows that both the enclosed bacteria and the threads produced by mutant 257DH4 are morphologically indistinguishable from those formed during the wild-type interaction between McCall and USDA191.

Acetylene reduction. We previously detected acetylene-reduction activity in roots inoculated with 257DH4, but not with 257DH5 (Heron et al. 1989). Table 1 quantifies such activity for 257DH4 and compares it to reference strain USDA191. Under growth-pouch conditions, the onset of acetylene-reduction activity in root systems inoculated with 257DH4 is somewhat slower than that in roots inoculated with USDA191. This difference, which is evident at day 12, reflects a relatively low rate of acetylene reduction by plants inoculated with the mutant and the fact that only 20% of such plants are nodulated at this time (compared to 90% of the plants inoculated with strain USDA191). By day 15 and thereafter, the rates are comparable (Table 1). Thus, for example, acetylene reduction by the mutant and by strain USDA191 was equivalent (0.42 versus 0.46 $\mu mol \cdot h^{-1}$ per plant) at three weeks after inoculation.

Nodulation blocking. Pairwise mixtures of 257DH4 and 257DH5 with strain USDA257 were used as inoculum in experiments to search for interactions between the mutants and the parental strain. Inclusion of the parental strain in inoculum containing 257DH5 had no effect on nodulation (2.3 versus 1.9 nodules per plant at 20 d for seedlings inoculated, respectively, with 257DH5 and the mixture), but USDA257 markedly influenced nodu-

Fig. 3A, B. Transmission electron microscopy of infection threads (*arrowheads*) in young soybean root nodules produced by **A** reference strain USDA191, and **B** mutant 257DH4; ×12500. Seedlings were dipped into bacterial suspensions containing 10^9 cells/ml and transferred to plastic growth pouches as described in *Material and methods*. Nodules were harvested 8–9 d after inoculation. *N* = nucleus; *CW* = cell wall. Bar = 2 μm

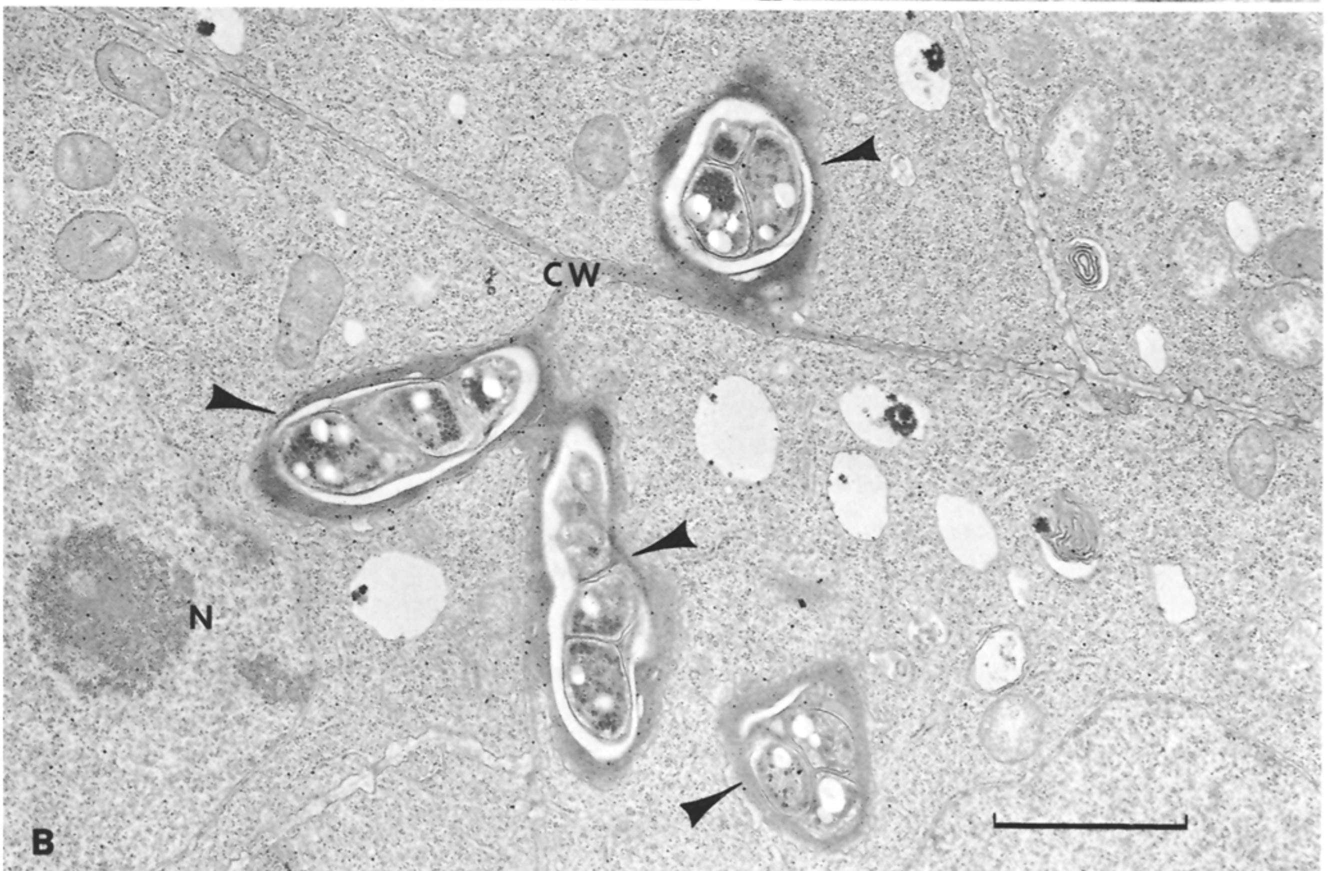
CW
N
B

Table 1. Acetylene-reduction by nodulated McCall soybeans. The total number of root systems examined by treatment in replicate experiments was 14–18. Values are means ± SE

Day	Strain USDA191		Mutant 257DH4	
	Acetylene reduction (μmol·h^{-1}·(g nodule)$^{-1}$)	Nodule mass (g·plant^{-1})	Acetylene reduction (μmol·h^{-1}·(g nodule)$^{-1}$)	Nodule mass (g·plant^{-1})
12	3.8 ± 0.8	7.8 ± 1.1	0.6 ± 0.3	5.2 ± 0.8
15	10.3 ± 1.9	10.7 ± 2.0	12.6 ± 2.8	7.5 ± 1.5
18	13.2 ± 1.6	10.9 ± 1.3	13.5 ± 1.6	12.5 ± 1.8
21	15.5 ± 0.9	26.9 ± 3.4	25.2 ± 2.4	20.2 ± 2.3

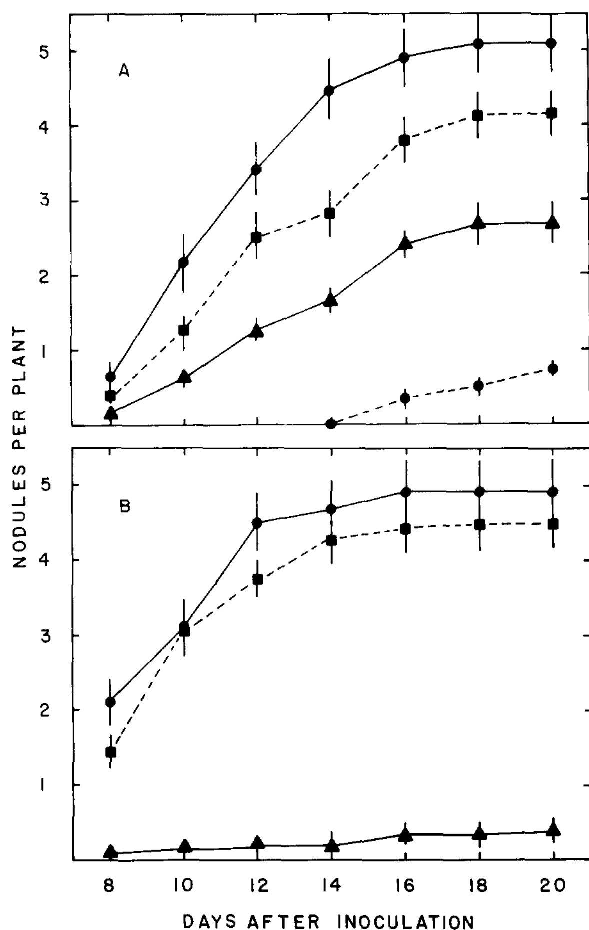

Fig. 4A, B. The parental strain, USDA257, of *R. fredii* blocks nodulation of McCall soybean by mutant 257DH4. **A** Concentration-dependence of the blocking response. Sets of seedlings were transferred to growth pouches and inoculated as described in *Material and methods* with suspensions containing mutant 257DH4 (10^9 cells/ml) and strain USDA257 at 0 cells/ml (●—●), 10^7 cells/ml (■---■), 10^8 cells/ml (▲—▲), or 10^9 cells/ml (●---●). **B** Effect of boiling strain USDA257 on the blocking response. Sets of seedlings were inoculated with suspensions containing mutant 257DH4 (10^9 cells/ml) and strain USDA257 at 0 cells/ml (■---■), 10^9 living cells/ml (▲—▲), or 10^9 boiled cells/ml (●—●). Each experiment was repeated twice with a total of 18 plants per treatment. Bars = SE

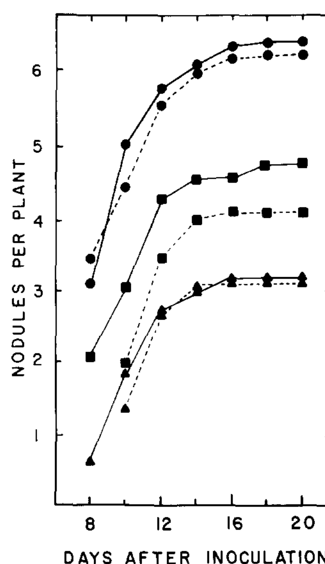

Fig. 5. Time- and dosage-dependence of the nodulation of McCall soybean by *R. fredii* 257DH4. Sets of seedlings in plastic growth pouches were inoculated with bacterial suspensions as described in *Material and methods*. Bacterial concentrations, in cells/ml, were as follows: ▲---▲, 10^4; ■---■, 10^5; ▲—▲, 10^6; ●—●, 10^7; ●---●, 10^8; ■—■, 10^9. Nodules were enumerated on alternate days between day 8 and day 20 after inoculation. The experiment was repeated, with a total of 40 plants per treatment. Least significant differences (LSD, 0.01) varied between 0.8 and 1.2 nodules/plant

lation by mutant 257DH4. At an inoculum concentration of 10^8 cells/ml, 257DH4 first produced nodules at 8 d after inoculation, and at day 20, seedlings averaged five nodules each (Fig. 4A). Inclusion of USDA257, even at a ratio of only about one cell per 10 cells of 257DH4, significantly reduced the number of nodules, and the magnitude of the blocking effect was greater if the cell ratio was 1:1 or 10:1 (Fig. 4A). In the latter case, the onset of nodulation was delayed by 6 d and the number of nodules reduced by 85%.

Several lines of evidence indicate that living cells of USDA257 are required for blocking. Boiled suspensions of USDA257 were unable to interfere with nodulation by 257DH4 (Fig. 4B). Filter-sterilized medium from USDA257 cultures was similarly inactive, as was a crude capsular polysaccharide fraction (Mort and Bauer 1980) from cells of USDA257 (data not shown). Blocking is not an artifact of the artificial growth-pouch system that was used to assess nodulation. This was confirmed by inoculating McCall seedlings as above with a mixture containing 10^9 cells of USDA257 and 10^8 cells of 257DH4/ml and planting the seedlings in Conetainers (Conetainer Nursery, Canby, Ore.), USA) (Pueppke

1986) containing vermiculite. Under these conditions, nodulation was reduced by 75% compared to that of controls that had received USDA257 alone (5.2 ± 0.3 versus 20.5 ± 1.9 nodules per plant, mean $\pm$ SE, respectively, at 16 d).

The blocking effect is not an anomalous consequence of supra- or suboptimal numbers of 257DH4 cells. This was confirmed by measuring the dependence of McCall nodulation on bacterial cell concentration (Fig. 5). It can be seen that nodulation was optimal at bacterial concentrations of 10^7–10^8 cells/ml, concentrations which span that used in the blocking experiments. Higher and lower bacterial cell numbers led to reduced nodulation, and very low bacterial cell concentrations (10^4–10^5/ml) delayed nodulation by 2 d (Fig. 5).

Discussion

Strains USDA191 and USDA257 typify the extremes of cultivar specificity in nodulation of soybean by *R. fredii*. Strain USDA191 nodulates primitive and advanced cultivars, and nitrogen is fixed (Keyser et al. 1982; Hattori and Johnson 1984; DuTeau et al. 1986; Israel et al. 1986). In marked contrast, strain USDA257 forms either abnormal Fix$^-$ nodules (Appelbaum et al. 1985c) or fails entirely in nodulating improved soybean cultivars (Heron and Pueppke 1984, 1987; Heron et al. 1989). On the basis of plasmid-transfer experiments, Appelbaum et al. (1985a, b, c) have argued that such cultivar specificity is encoded by the sym plasmid, but they were unable to demonstrate whether or not the relevent genes act positively or negatively. More recently, we identified two loci in strain USDA257 that regulate cultivar specificity in a negative fashion, i.e. disruption of the loci expands nodulation to new cultivars (Heron et al. 1989). One of these loci is chromosomal, and the other is on the sym plasmid. Insertional inactivation of either locus, as in mutants 257DH4 and 257DH5, permits the strain to nodulate the improved soybean cultivar McCall.

We now have characterized the cellular interactions of the two mutants with McCall soybean and compared them to those of wild-type strains USDA191 and USDA257. Inoculation with USDA191 led to the expected series of nodulation events, i.e. root-hair deformation, appearance of foci of meristematic cortical cells, infection-thread biogenesis, and ultimately, nodulation. These processes have been described in detail by Calvert et al. (1984) and need not be repeated here. Although strain USDA257 failed to produce nodules, it was not unreactive: Root hairs were stimulated to deform in a manner characteristic of compatible soybean-*Rhizobium* interactions (Turgeon and Bauer 1982; Pueppke 1983), and foci of dividing cortical cells appeared. Such cell division ceased at the stage of nodule-meristem formation, and infection threads were not observed. In soybean, neither root-hair curling nor the induction of meristematic activity in the cortex requires infection (Turgeon and Bauer 1982; Calvert et al. 1984). The fundamental constraint to nodule formation in the USDA257 McCall interaction thus seems to be failure of either infection-thread initiation or initial growth of threads. Detailed ultrastructural analysis of sites of apparent incipient infection will be required to distinguish between these alternatives.

Mutants 257DH4 and 257DH5 both are released from the factors that check infection by parental strain USDA257. Although infection proceeds in both cases, the end results differ. Nodules produced by mutant 257DH5 are abnormal and Fix$^-$ (Heron et al. 1989). The structure and development of nodules produced by mutant 257DH4 and reference strain USDA191, however, are qualitatively quite similar. Infection threads are common, and sustained cell divisions in foci give rise to Fix$^+$ nodules. Mutant 257DH4 produces fewer nodules per plant than does USDA191, and they appear later. Nodules produced by the mutant nevertheless fix more nitrogen on a nodule-weight basis. Acetylene-reduction rates of McCall roots nodulated by either USDA191 or by 257DH4 nonetheless are significantly less than those of roots nodulated by effective *Bradyrhizobium japonicum* strains (Israel et al. 1986).

The observation that strain USDA257 can block nodulation of McCall by mutant 257DH4 is very intriguing. The mutant originally was obtained from en-masse screening for rare Nod$^+$ exconjugants on McCall (Heron et al. 1989). Had blocking been absolute, such mutants could not have been recovered. Although similar interactions between other pairs of parental *Rhizobium* strains and derived Nod$^+$ mutants have not been sought (Djordjevic et al. 1985; Horvath et al. 1986), the situation with McCall bears striking resemblence to the response of Afghanistan pea to certain strains of *R. leguminosarum* bv. *viciae*. Afghanistan, a primitive cultivar, is effectively nodulated by the Turkish strain TOM, but it is not nodulated by the unrelated Dutch strain PF_2, which originally was selected for its superior ability to nodulate improved pea cultivars in Europe (Lie et al. 1978; Winarno and Lie 1979). If cells of PF_2 are added to an inoculum of TOM, nodulation of Afghanistan is either substantially delayed or abolished (Lie et al. 1978, 1988). Nodulation blocking in Afghanistan shares several important characteristics with the blocking that we have observed with McCall soybean: Both responses depend on the concentration of blocking cells, and such cells must be viable (Winarno and Lie 1979; Broughton et al. 1982; Lie et al. 1988). Moreover, PF_2, like USDA257, is capable of eliciting an initial response on the part of the plant. These responses differ in that PF_2 initiates visible infection threads (Lie 1984), but USDA257 does not. We nevertheless cannot exclude the possibility that USDA257 begins to infect, but that thread growth ceases before it can be resolved by light microscopy.

In spite of physiological similarity, there are important genetic distinctions between nodulation blocking in Afghanistan pea and that in McCall soybean. Strain TOM can nodulate Afghanistan because it contains a sym plasmid-borne gene, termed *nodX* (Davis et al. 1988). The ability of PF_2 to block nodulation, however,

is encoded by a 6.9-kilobase fragment that is distinct from *nodX* (Dowling et al. 1989). This fragment contains a series of *nod* genes, and at least three, *nodC, nodD*, and *nodE*, are required for nodulation-blocking activity (Dowling et al. 1989). Inactivation of *nodC* and *nodD* abolishes the nodulating ability of *R. leguminosarum* bv. *viciae* (Downie et al. 1985). Strain PF_2 consequently seems to employ a single set of genes to both nodulate improved pea cultivars and block nodulation of a primitive cultivar by an unrelated strain.

In contrast, a single Tn*5* insertion into the sym plasmid of *R. fredii* USDA257 appears simultaneously to expand host range to soybean cultivar McCall and to render such nodulation sensitive to blocking by the non-mutated parental strain. Disruption of *nodDABC* seems not to be involved in these changes (Heron et al. 1989). One possibility is that as the frequency of unproductive infections by USDA257 increases with its increasing share of the inoculum, the number of productive infections initiated by the mutant declines. In previous experiments, however, we found that nodulation of McCall by USDA191 is not similarly sensitive to the presence of USDA257 (Heron and Pueppke 1987). Thus, blocking of nodulation by a second strain cannot be just a generalized plant regulatory response to unproductive infections.

One hypothesis to explain our observations is that the gene or genes disrupted in mutant 257DH4 encode a product(s) that halts nodulation of McCall after the onset of cortical cell divisions but prior to infection per se. The absence of this product(s) in mutant 257DH4 allows infection to proceed, but nodulation remains sensitive to the product(s), which can be supplied by cells of USDA257, if they are included in the inoculum. Testing of this hypothesis will require coordinated genetic analysis of mutant 257DH4 and detailed physiological characterization of responses of the rhizobia and plant to one another. Both are currently underway in our laboratory.

This research was underwritten by Grant No. 88-37234-4101 from the U.S. Department of Agriculture. Asita Chatterjee was supported by funds from the Food for the 21st Century Program of the University of Missouri, and Pedro Balatti was supported by the Consejo Nacional de Investigaciones Cientificas Tecnicas de la Republica Argentina. We thank W.J. Broughton for providing facilities during preparation of the manuscript. This is Journal Series No. 10857 of the Missouri Agricultural Experiment Station.

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Received 4 July; accepted 19 October 1989