Regulation of Nodulation in the Soybean-*Rhizobium* Symbiosis[1]

STRAIN AND CULTIVAR VARIABILITY

DAVID S. HERON*[2] AND STEVEN G. PUEPPKE
Department of Plant Pathology, University of Missouri, Columbia, Missouri 65211

ABSTRACT

Double inoculation (15 h apart) of the soybean cultivar Williams with *Bradyrhizobium japonicum* I-110ARS reveals a rapid regulatory plant response that inhibits nodulation of distal portions of the primary root (M Pierce, WD Bauer 1984 Plant Physiol 73: 286–290). Only living, homologous rhizobia elicit the response. We conducted similar double inoculation experiments to test the hypothesis that this is a universal phenomenon in soybean symbioses. We investigated interactions of the cultivar McCall with the slow-growing strain *Bradyrhizobium* sp. 3185 (= 3G4b16) and strains of the fast-growing soybean symbiont, *Rhizobium fredii* (USDA191 [Nod⁺ on McCall] and USDA257 [Nod⁻ on McCall]). Nodulation was not detectably inhibited when USDA257 was included in various combinations with an inoculum of USDA191. Strain USDA257 cohabited nodules with strain USDA191 when plants were inoculated sequentially with both strains, but USDA257 did not nodulate McCall when a sterile culture filtrate of USDA191 was added to USDA257 inoculum. There was only a slight inhibition of nodulation of distal portions of the primary root in double inoculation experiments with McCall and strain 3185. Because these results were unexpected, we repeated the experiments with Williams and strain I-110ARS. The response was similar to that observed in the McCall × 3185 interaction. Regulation of nodulation on the primary root thus appears to be variable and depend on strain × cultivar interactions.

The symbiotic association between soybean and rhizobia enables the bacteria to fix atmospheric nitrogen while living in the protective environment of a root nodule. Establishment of the symbiosis proceeds through a developmental sequence that is under control of both symbiotic partners. Regulation is a poorly understood phenomenon in all *Rhizobium*-legume symbioses. In clover and soybean, there are often more infections than resultant nodules (4, 10), and in some cases the response is strain × cultivar-specific (7). Position of nodules on the root system also appears to be regulated in some unknown fashion. Soybean root nodules commonly are distributed near the crown of the plant under field conditions (18). In studies of the mechanisms of regulation of nodulation, plants often are grown under more controlled conditions, *e.g.* plastic growth pouches. Nodulation of pouch-grown soybean typically is monitored on a restricted portion of the root system of seedlings (usually for <10 d after

inoculation). Nodules frequently are clustered on proximal portions of the primary roots of such plants, but some strain × cultivar interactions produce almost no clustering (2, 3, 7, 14). Using such a system with soybean, Pierce and Bauer (11) reported the existence of a rapid regulatory response that inhibits nodulation of distal portions of the primary root following inoculation of seedlings with *Bradyrhizobium japonicum*. The response is not elicited by heterologous rhizobia or dead *B. japonicum* cells.

We partially characterized 11 unusual strains of soybean rhizobia (7) that were recovered from soils of the People's Republic of China (8). The physiological and genetic differences between these strains and the typical soybean rhizobia, *B. japonicum*, are sufficient to classify them in a separate species, *Rhizobium fredii* (15). The *R. fredii* strains exhibit cultivar-specific nodulation of soybean genotypes, an unusual trait among soybean rhizobia (7). We chose two *R. fredii* strains, USDA191 and USDA257, and McCall soybean to extend the findings of Pierce and Bauer (11). In particular, we wanted to test the generality of the phenomenon that limits distal nodulation, and also determine if a plant regulatory response would be elicited by USDA257, a strain that does not nodulate McCall but nodulates other soybean genotypes.

MATERIALS AND METHODS

Organisms and Growth Conditions. Soybean seeds (*Glycine max* [L.] Merr.) cv McCall were obtained from D. Whited, North Dakota State University, Fargo. *Rhizobium fredii* strains USDA191 and USDA257 (hereafter referred to as 191 and 257) and *Bradyrhizobium* sp. 3185 (= 3G4b16) were from H. H. Keyser, United States Department of Agriculture, Beltsville, MD. Strain 3185, a slow-growing strain isolated originally from peanut (*Arachis hypogaea* L.), also nodulates soybean (12, 13). Both strains 191 and 3185 produce wild-type, pink-to-red-colored root nodules on cv McCall (DS Heron, SG Pueppke, unpublished observations). W. D. Bauer (Ohio State University, Columbus) provided seeds of soybean cv Williams and a lyophilized stock of *Bradyrhizobium japonicum* I-110ARS. Strains 191, 257, and 3185 were kept in long-term storage in YEM[3] broth (17) plus 7% glycerol at −80°C. Short-term storage was on YEM agar at 10°C. For inoculations, strains 191, 257, and 3185 were grown to midlog phase in YEM broth at 30°C and 125 rpm. Strain I-110ARS was grown as described previously (11). Seeds were surface-disinfested and germinated on water agar in the dark. Groups of three seedlings were grown in sterilized plastic pouches (Northrup-King, Minneapolis, MN) containing N-free, Jensen's solution (17). Plants were given deionized water as needed and maintained at 25°C under a 12-h photoperiod with a light intensity of 500 μE/m²·s (photosynthetically active radiation).

Nodulation Experiments. The basic protocol of Pierce and

[2] Present address: USDA-ARS, Root Disease and Biological Control Unit, 367 Johnson Hall, Washington State University, Pullman, WA 99164-6430.

[3] Abbreviations: YEM, yeast extract-mannitol; RDU, relative distance unit.

Bauer (11) was followed. Pouch-grown seedlings (2-3 d old) were inoculated twice, 15 h apart. Seedlings were inoculated with 250 μl of bacteria suspended in half-strength Jensen's solution, or 250 μl of a sham inoculum (half-strength Jensen's solution) at each of the inoculation times. The positions of the primary root tip at the time of the first and second inoculations were marked on the pouch as RT1 and RT2, respectively. The distance between the marks defines the growth rate of the root, and is defined as one RDU to normalize the distance data for plants with different growth rates. Such normalization is important, because the zone of infectible cells progresses acropetally behind the growing root tip (2). Positions of primary root nodules relative to the RT1 mark were recorded 7 and 10 d after the first inoculation.

In experiments with McCall and the R. fredii strains, the protocol was modified to omit the PBS wash of the bacteria, and the 24-h delay between placement of the seedlings into the pouches and inoculation. Preliminary experiments indicated no difference in nodulation responses whether the original or modified protocols were used. We conducted two series of double inoculation experiments with McCall and R. fredii: one series with the small inoculum dose of 10^5 bacteria/ml as used in previous studies (11), and one series with a large inoculum dose of 10^8 bacteria/ml.

The number of plants per treatment per experiment varied from 9 to 18. Data from repeated experiments were pooled for analysis. Sham-inoculated treatments were included in each experiment. Plants receiving both first and second inoculations with sham inoculum never developed nodules, nor did plants receiving inoculations with strain 257 only, or strain 257 with sham inoculum. A single set of cultures was used for the inoculation treatments within each experiment. Inoculum concentrations were adjusted turbidimetrically and confirmed by plating on YEM agar. In experiments with cv Williams and strain I-110, we followed the unmodified protocol described above (PBS wash of cells; 24 h delay between placing seedlings in pouch and inoculation) and recorded primary root nodule positions 7 d after the first inoculation.

Identification of Nodule Occupants. Indirect immunofluorescence microscopy was used to identify R. fredii strains from nodules. Anti-257 serum for these assays was produced in New Zealand White rabbits according to the procedure outlined by Vincent (17). Preimmune serum was used for controls. Anti-191 serum was provided by H. P. Friedman, University of Missouri-St. Louis. Gamma-globulin fractions of antisera were obtained by ammonium sulfate precipitation and stored at −20°C.

Surface-sterilized nodules were crushed and three droplets of bacteria were placed on a microscope slide. After heat fixation of the bacteria, the slides were dipped in PBS, and air-dried. Droplets of a 1:10 dilution of the primary antibody were placed on the fixed cells and incubated for 15 min in a moist chamber. The slide then was dipped briefly in PBS before immersion in PBS for 5 min. Excess moisture was removed before addition of a droplet of the secondary antibody, a 1:25 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (Sigma Chemical Co.), and incubation for 15 min in a moist chamber. The slide was then dipped in PBS before immersion in PBS for 15 min. A drop of mounting medium (equal parts PBS and glycerol) was added before application of the cover slip. Cells were observed with an Olympus model BH2 microscope equipped with a B-L0893 epifluorescence system (exciter = BP-490, dichroic mirror = DM500, barrier = 0-515). The rhizobia from each nodule were tested with anti-191, anti-257, and preimmune antibodies. Preliminary testing of the antisera developed against the R. fredii strains indicated that strains 191 and 257 are serologically distinct.

Experiments with Bacterial Culture Filtrate. These experi-

ments were designed to determine if culture filtrates of strain 191 could render strain 257 competent to infect and nodulate McCall. Bacteria were grown in yeast extract-sucrose broth (YEM with sucrose substituting for mannitol; strains grow equally well in either medium). Log phase cultures of strains 191 and 257 were harvested by centrifugation. The supernatant solution from strain 191 was passed twice through 0.45 μm filters to obtain the sterile filtrate. Aliquots were plated to confirm sterility. Cells were resuspended in half-strength Jensen's solution or filtrate, and adjusted to 1-5 × 10^9 bacteria/ml. Two-d-old seedlings were inoculated with 250 μl of one of the following: strain 257, strain 257 in filtrate, cell-free filtrate, strain 191, or a sham inoculum of half-strength Jensen's solution. Nodulation and infection experiments each were repeated once with a total of 18 and 12 plants per treatment, respectively. Nodulation was recorded 21 d after inoculation. Plants were scored for infection threads 6 to 9 d after inoculation as described previously (12).

RESULTS

Nodulation of McCall by *Rhizobium fredii*. In preliminary experiments we directly adapted the double inoculation protocol used by Pierce and Bauer (11) to study regulation of nodulation in interactions of McCall with R. fredii strains. We began with the relatively small inoculum (10^5 bacteria/ml) used earlier with B. japonicum and scored plants for nodulation 7 and 10 d after the first inoculation. The low inoculum level produced averages of 2.5 to 4.6 nodules/plant by 7 d. Nodule numbers increased to 5.0 to 7.4 nodules/plant by 10 d after the first inoculation. Very few new primary root nodules appeared after 10 d. The nodulation profiles in these experiments unexpectedly lacked the discrete peaks that characterized the nodule clustering in previous studies (11, 16). These findings suggested that the inoculation conditions may have been suboptimal for R. fredii and thus prompted us to try to optimize the nodulation response for McCall and strain 191.

Pierce and Bauer (11) optimized the nodulation response for B. japonicum by determining the inoculum concentration that yielded (a) the maximum number of primary root nodules/plant, (b) the highest percentage of nodules above the RT mark, and (c) the greatest percentage of plants with nodules above the RT mark in single inoculation studies. Similarly, we investigated nodulation of McCall in response to inoculation with 250 μl of R. fredii concentrations ranging from 10^4 to 10^8 cells/ml. Nodulation responses for each of the three parameters increased with increasing inoculum concentration and plateaued at 10^8 cells/ml.

In double inoculation experiments, the shapes of the nodulation profiles for each of the treatments were virtually the same for small and large inoculum doses. When the smaller inoculum was used, the profiles resembled those shown in Figure 1, except that the overall heights of the profiles were slightly reduced (fewer nodules per plant, Table I). Small and large inoculum regimes had little or no effect on the mean distance of primary root nodules from the RT1 at 7 d after the first inoculation (Table I). Regardless of the inoculum level, plants continued to develop nodules up to 10 d after the first inoculation, after which very few new nodules appeared. On the basis of these preliminary experiments, we chose 10^8 cells/ml as the optimal inoculum concentration for use in the remainder of the experiments with McCall, and we recorded nodulation 10 d after the first inoculation.

The distribution of primary root nodules on McCall in response to strains 191 and 257 is given in Figure 1. Because of natural differences in root growth rate among different plants, data were normalized in the manner recommended by Pierce and Bauer (Fig. 1 in Ref. 11). Distances of nodules from RT1 were converted to RDU, and the number of nodules per 0.2
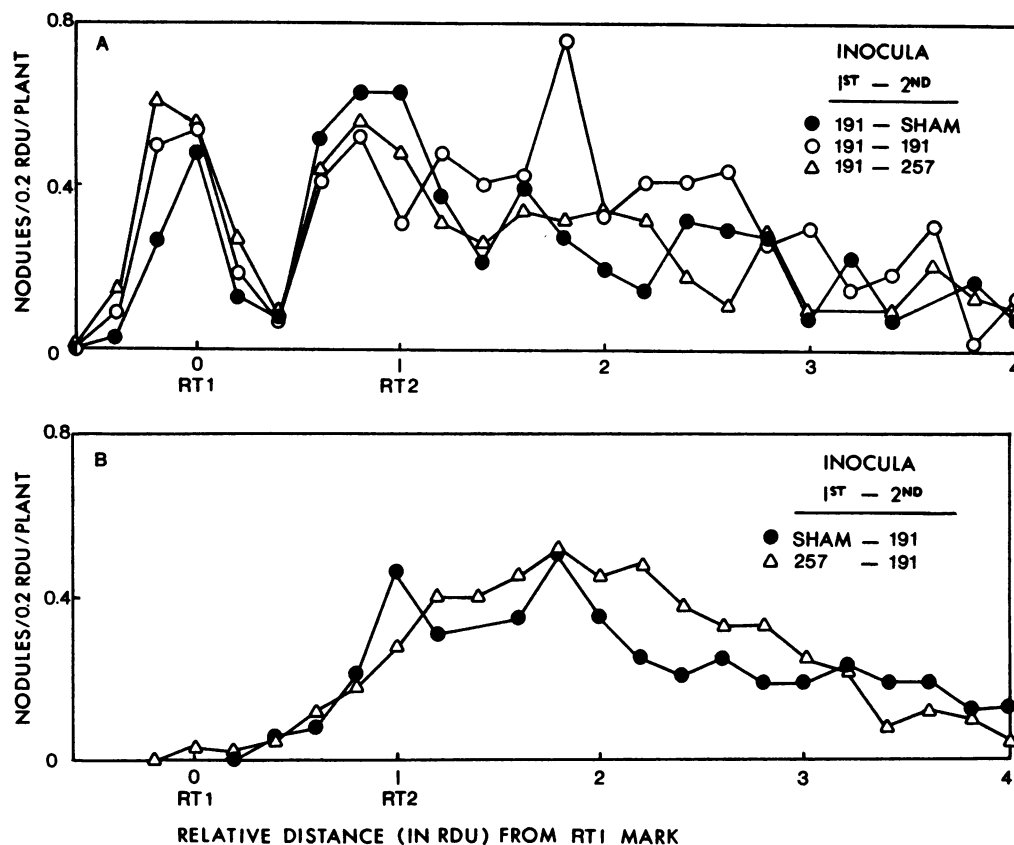
FIG. 1. Distribution of primary root nodules on McCall soybean seedlings inoculated twice, 15 h apart, with *R. fredii* strains 191 and 257. The position of the root tip at each time of inoculation was marked on the surface of the pouch (first inoculation as RT1; second inoculation as RT2). Each seedling received 250 μl of a bacterial suspension (10^8 cells/ml) or sterile half-strength Jensen's solution (sham inoculation) at each inoculation time. Distances of nodules from the RT1 mark are given in RDU as described in "Materials and Methods." Data are pooled from six repeated experiments for each treatment. The total number of plants per treatment ranged from 50 to 62. The direction of root growth is from left to right.

Table I. *Nodulation of McCall Soybean in Response to Double Inoculations with R. fredii Strains 191 and 257*

Inoculum[a] (First/Second)	Mean No.		Mean Nodule Distance	
	Small inoculations	Large inoculations	Small inoculations	Large inoculations
	primary root nodules/plant $\pm$ *SE*[b]		*RDU from RT1* $\pm$ *SE*[c]	
191/Sham	3.4 $\pm$ 0.4	4.8 $\pm$ 0.3	1.8 $\pm$ 0.1	1.4 $\pm$ 0.1
191/191	3.6 $\pm$ 0.5	5.5 $\pm$ 0.4	1.5 $\pm$ 0.1	1.5 $\pm$ 0.1
191/257	4.6 $\pm$ 0.5	5.0 $\pm$ 0.4	1.5 $\pm$ 0.1	1.2 $\pm$ 0.1
Sham/191	2.5 $\pm$ 0.4	3.6 $\pm$ 0.3	2.4 $\pm$ 0.1	2.2 $\pm$ 0.1
257/191	2.6 $\pm$ 0.3	3.9 $\pm$ 0.3	2.6 $\pm$ 0.1	2.1 $\pm$ 0.1

[a] The interval between the first and second inoculations was 15 h. Sham inoculations consisted of 250 μl of half-strength Jensen's solution. [b] Small and large inoculum doses consisted of 250 μl of 10^5 bacteria/ml and 250 μl of 10^8 bacterial/ml, respectively. Numbers of nodules were recorded 7 d after the first inoculation. The number of plants per treatment ranged from 21 to 25 (small inoculations) and from 62 to 75 (large inoculations). [c] One RDU = distance the root tip traverses between inoculation times.

RDU was plotted (RDU values ranged from 8 to 39 mm; mean = 22 mm; SD = 8). The three nodulation profiles in Figure 1A are for treatments with strain 191 as the first inoculum. All have peaks of nodules clustered about the RT1 mark and conspicuous valleys between the RT1 and RT2 marks. Nodulation resumes just above the RT2 mark and continues for more than 4 RDU. This distance is equivalent to 60 to 120 mm below the RT1 mark, a position at or near the bottom of the pouch. With the exception of the valley between RT1 and RT2, nodule numbers gradually decreased toward distal portions of the root. The use of strain 191, strain 257, or a sham inoculum for the second inoculation did not obviously influence the shape of the profile.

Figure 1B contains the nodulation profiles from plants that received strain 191 for the second inoculation. Although distinct from the shapes in Figure 1A, the profiles are virtually the same whether the first inoculation consisted of strain 257 or a sham

inoculum. The distribution of nodules is very broad, spanning over 4 RDU with a peak in the vicinity of one RDU from the RT2 mark. The lack of a peak at RT1 when 257 was the first inoculum suggests that nodulation was due to strain 191.

Nodulation of Williams by *B. japonicum* I-110ARS. The shapes of the nodulation profiles from interactions involving *R. fredii* strains were markedly and unexpectedly different from the relatively sharp profiles reported for interactions of soybean with *B. japonicum* strains (11, 16). In an effort to resolve these differences, we repeated the experiments of Pierce and Bauer (11). Nodulation profiles from these experiments are given in Figures 2 and 3. Primary root nodules appeared several days sooner after inoculation in this interaction than in that of McCall and strain 191, and few new nodules appeared after 7 d following inoculation. Plants inoculated first with strain I-110 ARS and then with sham inoculum yielded profiles with the expected peak
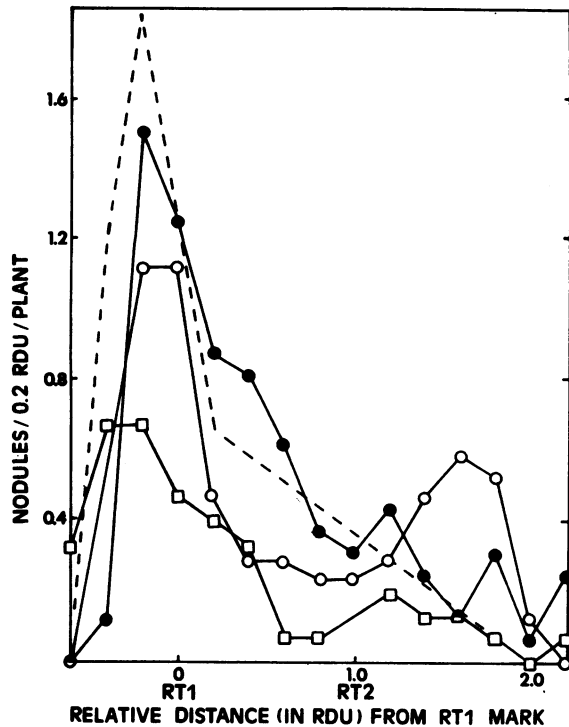
FIG. 2. Variability in the distribution of primary root nodules on Williams soybean seedlings inoculated twice, 15 h apart, with *B. japonicum* I-110ARS then a sham inoculum of half-strength Jensen's solution. Data from three repeated experiments (O, ●, □) are plotted. Data were obtained from 14 to 17 plants for each repeated experiment. The broken line represents an approximation of the data from an earlier experiment (Fig. 1 of Ref. 11). Distances of nodules from the RT1 mark are given in RDU as described in "Materials and Methods." The direction of growth is from left to right.

of nodules in the vicinity of the RT1 mark, but there was experiment to experiment variability in the heights of the peaks (Fig. 2).

The data from three repeated experiments were pooled to obtain the nodulation profiles shown in Figure 3. Plants that received strain I-110ARS for the first inoculation and a sham inoculum for the second inoculation yielded a profile with the expected prominent peak centered about the RT1 mark. Plants doubly inoculated with strain I-110ARS produced a profile with a moderate peak that is centered about the RT1 mark and declines slightly in the vicinity of the RT2 mark. Plants sham-inoculated and then inoculated with strain I-110ARS generated profiles with a moderate peak centered about the RT2 mark and very few nodules above the midpoint between RT1 and RT2. Few nodules were found more than 2.1 RDU from the RT1 mark in any treatment.

Nodulation of McCall by *Bradyrhizobium* sp. 3185. Almost all infections give rise to nodules in McCall × 191 interactions in growth pouches (7). In contrast, both Williams × I-110ARS and McCall × 3185 interactions result in many surplus infections that never yield nodules (4, 7). We exploited this distinction to test the influence of surplus infections on the regulation of nodulation, specifically asking whether strain 3185 would elicit in McCall a regulatory response similar to that described for the Williams × I-110ARS interaction (11). Plants inoculated first with strain 3185 and then with sham inoculum yielded a profile with a broad peak of primary root nodules at the RT1 mark followed by a gradual decline in nodules (Fig. 4). This profile is nearly identical to that derived from plants receiving a double inoculation with strain 3185. Plants inoculated first with a sham inoculum and then with strain 3185, however, displayed a profile with a rather sharp peak just above the RT2 mark followed by a fairly sharp dropoff. Primary root nodulation in each of the treatments tapered to low levels within 2 RDU of RT1.

Table II provides some additional comparisons among the nodulation responses of McCall and Williams. Within analogous treatments, the mean distance of primary root nodules from the RT1 mark is very similar for McCall × 3185 and Williams × I-110ARS interactions. This distance is consistently greater in McCall × 191 interactions. Nearly all of the interactions and treatments produced comparable numbers of primary root nod-
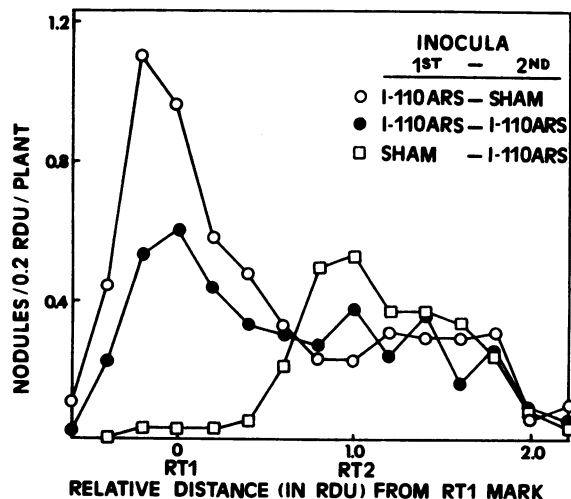

FIG. 3. Distribution of primary root nodules on Williams seedlings inoculated twice, 15 h apart, with *B. japonicum* I-110ARS or a sham inoculum. Distances of nodules from the RT1 mark are given in RDU as described in "Materials and Methods." Data are pooled from three repeated experiments. The total number of plants per treatment ranged from 37 to 46. The direction of root growth is from left to right.
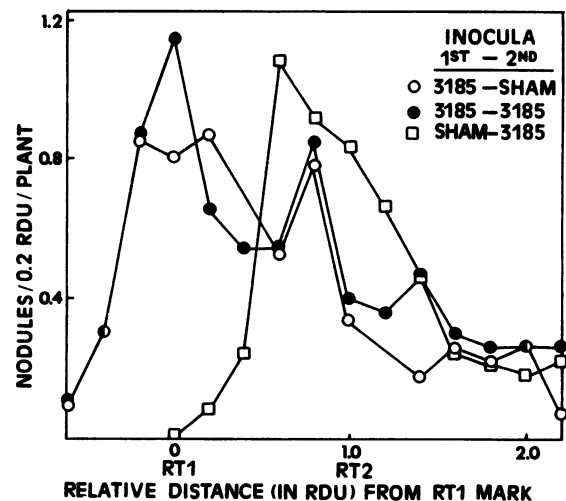

FIG. 4. Distribution of primary root nodules on McCall seedlings inoculated twice, 15 h apart, with *Bradyrhizobium* sp. 3185 or a sham inoculum. Distances of nodules from the RT1 mark are given in RDU as described in "Materials and Methods." Data are pooled from four repeated experiments. The total number of plants per treatment ranged from 42 to 47. The direction of root growth is from left to right.

Table II. *Nodulation of McCall and Williams Soybean in Response to Double Inoculations*

Each seedling was inoculated twice, 15 h apart, with 250 μl of 10^8 bacteria/ml (McCall), 10^5 bacteria/ml (Williams), or sham inoculum (sterile half-strength Jensen's solution). Nodule positions were recorded 7 d (Williams) or 10 d (McCall) after the first inoculation. Data for McCall × 191 interactions are from 50 to 62 plants in six experiments. Data for McCall × 3185 interactions are from 42 to 47 plants in four experiments. Data for Williams interactions are from 37 to 46 plants in three experiments.

Host	Inoculum (First/Second)	Mean No.	Mean Nodule Distance
		primary root nodules/ plant ±SE	*RDU from RT1 ±SE*[a]
McCall	191/Sham	6.4 ± 0.4	1.8 ± 0.1
McCall	3185/Sham	7.4 ± 0.6	0.7 ± 0.1
Williams	I-110ARS/Sham	6.3 ± 0.5	0.5 ± 0.1
McCall	191/191	8.4 ± 0.5	1.9 ± 0.1
McCall	3185/3185	8.3 ± 0.6	0.8 ± 0.1
Williams	I-110ARS/I-110ARS	5.0 ± 0.4	1.1 ± 0.1
McCall	Sham/191	5.6 ± 0.4	2.6 ± 0.1
McCall	Sham/3185	6.8 ± 0.6	1.7 ± 0.1
Williams	Sham/I-110ARS	3.6 ± 0.4	1.5 ± 0.1
McCall	257/191	6.4 ± 0.4	2.6 ± 0.1
McCall	191/257	7.1 ± 0.5	1.6 ± 0.1

[a] One RDU = distance the root tip traverses between inoculation times.

ules per plant, with the exception of treatments in which Williams was sham-inoculated first and then inoculated with strain I-110ARS. The number of primary root nodules per McCall plant was slightly higher in treatments when strain 257, rather than sham inoculum, was paired with inoculation with strain 191. This prompted us to determine if strain 257 is able to nodulate McCall when strain 191 was in the rhizosphere.

Identification of Nodule Occupants. Plants inoculated with both strains 191 and 257 in three double inoculation experiments were used for the serological identification of nodule rhizobia. A total of 84 nodules from 59 plants were examined. In all cases nodule bacteria reacted with anti-191 antibody. Of the 76 nodules examined from 49 plants that were coinoculated with strains 191 and 257, 29 nodules had bacteria that reacted with anti-257 antibody. Based on rough visual estimations, strain 257 appeared to comprise less than 10% of the rhizobia in 21 nodules, approximately 10% of the cells in 7 nodules, and about 50% in one nodule. Strain 257 was never the sole bacterial inhabitant of a nodule.

Experiments with Bacterial Filtrate. Addition of culture filtrate from strain 191 to strain 257 cells did not enable the cells to initiate either infection threads (12) or nodules on McCall. The microscopic appearance of strain 257-inoculated plants was indistinguishable from that of plants inoculated with strain 257 in culture filtrate of strain 191. These plants had areas exhibiting root hair curling, golden discoloration, and dividing subepidermal cells typical of a compatible interaction (DS Heron, SG Pueppke, unpublished observations), but infection threads were absent and nodules did not develop.

DISCUSSION

Regulation of nodulation is a well known but poorly understood phenomenon in *Rhizobium*-legume symbioses. Nodules in effective N-fixing interactions are clustered on primary and secondary roots near the crown; large numbers of nodules are scattered on secondary and tertiary roots in ineffective symbioses (1). Distribution of root nodules also is influenced by strain and cultivar interactions in field-grown soybean (6, 18). Grubinger *et al.* (6) reported that depth of nodules from the soil surface differed among cultivars, with the percentage of nodules below 15 cm

ranging from 25 to 86%. Under the more controlled conditions of plastic growth pouches, nodule distribution patterns also appear to be dependent on strain × cultivar interactions (7, 14).

Inoculation of soybean seedlings with compatible rhizobia suppresses nodulation in response to subsequent inoculation with compatible rhizobia. Using sand culture for a split-root system, Kosslak and Bohlool (9) reported an apparently translocatable suppression when the interval between inoculation of the two portions of the root system was at least 4 d (the minimum time interval they studied). Studying young (<10 d) soybean seedlings grown in plastic pouches, Bhuvaneswari et al. (2) determined that nodulation of the primary root is developmentally restricted to the zone of immature cells just behind the growing root tip. As this zone moves acropetally, the infectibility of maturing cells is reduced so that the resultant nodulation profile forms a sharp peak near the original site of the root tip and then rapidly declines to a low level on distal portions of the primary root (2, 4). Bhuvaneswari et al. (2) hypothesized that the diminution of distal nodulation might be controlled by a fast acting regulatory mechanism of the host. Pierce and Bauer (11) tested this hypothesis by supplying seedlings with a second inoculation 15 h after the first. Inoculation with homologous rhizobia attenuates nodulation of distal portions of the primary root, even when additional inoculum is provided to the new region. The response is not elicited by heterologous rhizobia or dead *B. japonicum* cells. The sharply delineated nodulation profiles indicate that the plant responds quickly to inhibit nodulation of distal portions of the primary root. Takats (16) conducted similar double inoculation experiments with strain I-110ARS and cultivar Pride and used a 10 h interval between inoculations. Although there was significant overlap among profiles from different treatments, he concluded that his data agreed with the previously hypothesized rapid regulatory response.

We sought to test whether regulation of nodulation in the interaction of McCall with *R. fredii* 191 is similar to that described by Pierce and Bauer (11), and if so, whether the homologous, but nonnodulating strain 257 would elicit the regulatory response. Strain 257 forms normal infection threads and nodules on some cultivars of soybean (7). Although 257 does not form infection threads or nodules on McCall, it does induce root hair

curling and the initiation of foci of cell divisions in the subepidermal tissue beneath some of the curled root hairs (DS Heron, SG Pueppke unpublished data). The response appears similar to the pseudoinfections described by Calvert et al. (4). Thus, the response of plants to inoculation with strain 257 prior to inoculation with strain 191 might delineate the relative importance of preinfection events in eliciting the regulatory response.

It is difficult to compare the nodulation responses reported in previous double inoculation studies because the data have been normalized and plotted differently. The RDU has been defined as the distance between the root tip and the smallest emergent root hair (16, Fig. 2 in Ref. 11) or as the distance of root growth between the times of the first and second inoculations (Fig. 1 in Ref. 11). The two definitions yield very different values for the RDU. For example, cv Pride roots grew 3.4 RDU in the 10 h between dual inoculations (16). This value would be 1 RDU if the alternative definition is used. Our nodule distribution graphs have been prepared as those in Figure 1 of Ref. 11, to facilitate comparison with previous findings.

Unlike the profiles reported in studies employing I-110ARS (11, 16), clustering was not apparent in McCall × 191 interactions. Nodulation profiles were remarkably similar among all treatments in which the first inoculum was 191 (Fig. 1A). The valley in the profile between RT1 and RT2 was consistent across experiments regardless of inoculum concentration or delayed inoculation for 24 h after seedlings were placed in the pouches. This decrease in nodules in a narrow band between the RT1 and RT2 marks may be an artifact of the inoculation procedure; no decrease was observed in McCall seedlings dip-inoculated once with strain 191 (7). Regardless of the interpretation on this point, it is apparent that the interaction of McCall and 191 does not exhibit a rapid regulatory response that limits distal nodulation.

The effect of strain 257 on nodulation appeared to be no different than that of the sham inoculum, having no apparent inhibitory effect on nodulation in the two treatments where strain 191 was the other inoculum. The slight stimulation of nodulation in these treatments prompted us to identify nodule occupants. We wanted to determine if the presence of strain 191 in the rhizosphere enables strain 257 to nodulate McCall in a manner similar to that described by Devine et al. (5) for an antibiotic-resistant, nonnodulating strain of B. japonicum. Cells of strain 257 were detected in nearly 40% of the nodules from dually inoculated plants. The ability of strain 257 to enter the nodules seems to require the presence of 191 cells, presumably to induce infection thread formation.

Because of the disparity between our results with McCall × 191 and the results of Pierce and Bauer (11) with Williams × I-110ARS, we repeated their double inoculation experiments using seeds and a culture of I-110ARS provided by Dr. Bauer. Our results agree in part (Fig. 2) with the previous findings, which appear to be based on a single experiment (Fig. 1 in Ref. 11). However, there was considerable variation among our repeated experiments (Fig. 2). When the data from three such experiments are combined in nodulation profiles, the regulatory response of plants doubly inoculated with strain I-110ARS (Fig. 3) is not as dramatic as that previously reported (11). Takats (16) used strain I-110ARS with Pride soybean in similar experiments and obtained nodulation profiles that also differ from those reported by Pierce and Bauer (11). Variation in the numbers of nodules elicited among repeated experiments also seems evident if one compares the y-axes of Figures 1 and 2 in Ref. 11.

Pierce and Bauer (11) suggested that the strength of the regulatory response was directly correlated to the numbers of B. japonicum nodules above the RT1 mark. Visual inspection of their Figures 1 and 2 and our Figure 1A shows that the numbers

of such nodules are comparable for both interactions, thus making the correlation questionable for R. fredii. We sought to test whether the regulatory response might be activated instead by infection thread formation. In previous work with pouch-grown plants, we found that the ratio of infections to nodules for strain 191 × McCall is 1.2, whereas the ratio in interactions of McCall × Bradyrhizobium sp. strain 3185 is 46.8. The strains induce comparable numbers of nodules, and thus many of the 3185 infections are surplus. The experiments with McCall and strain 3185 yielded nodulation profiles with more pronounced clustering than in the McCall × 191 interactions, but there is no evidence of a rapid regulatory response inhibiting distal nodulation (Fig. 4). Any inhibition amounts to less than one nodule per plant (Table II, Fig. 4). It also is interesting to note that the McCall × 3185 interaction is more like the Williams × I-110ARS interaction than the McCall × 191 interaction. Both strains I-110ARS and 3185 are slow-growing rhizobia, whereas strain 191 is a member of the separate fast-growing species, R. fredii. Similarities in nodulation responses involving strains I-110ARS and 3185 may be associated with the potential of these strains to form large numbers of surplus infections on roots of pouch-grown seedlings.

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