Plant and Soil 108, 211–219 (1988)

PLSO 7488

Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia

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Received 6 August 1987. Revised November 1987

Key words: antibiotics, biological control, Bradyrhizobium, Rhizobium

Abstract

A study was conducted to determine whether colonization of legume roots and nodulation by *Rhizobium meliloti* and *Bradyrhizobium japonicum* could be enhanced by using inocula containing microorganisms that produce antibiotics suppressing soil or rhizosphere inhabitants but not the root-nodule bacteria. An antibiotic-producing strain of Pseudomonas and one of Bacillus were isolated, and mutants of *R. meliloti* and *B. japonicum* sp. resistant to the antibiotics were used. The colonization of the alfalfa rhizosphere and nodulation by *R. meliloti* were enhanced by inoculation of soil with *Pseudomonas* sp. in soil initially containing 2.7×10^5 *R. meliloti* per g. The colonization of soybean roots by *B. japonicum* was enhanced by inoculating soil with three cell densities of *Bacillus* sp., and nodulation was stimulated by *Bacillus* sp. added at two cell densities. In some tests, the dry weights of soybeans and seed yield increased as a result of these treatments, and co-inoculation with Bacillus also increased pod formation. Inoculation of seeds with *Bacillus* sp. and the root-nodule bacterium enhanced nodulation of soybeans and alfalfa, but colonization by *B. japonicum* and *R. meliloti* was stimulated only during the early period of plant growth.

Studies were also conducted with *Streptomyces griseus* and isolates of *R. meliloti* and *B. japonicum* resistant to products of the actinomycete. Nodulation of alfalfa by *R. meliloti* was little or not affected by the actinomycete alone; however, both nodulation and colonization were enhanced if the soil was initially amended with chitin and *S. griseus* was also added. Chitin itself did not affect *R. meliloti*. Treatments of seeds with chitin or *S. griseus* alone did not enhance colonization of alfalfa roots by *R. meliloti* or soybean roots by *B. japonicum*, but the early colonization of the roots by both bacterial species was promoted if the seeds received both chitin and *S. griseus*; this treatment also increased nodulation and dry weights of alfalfa and soybeans and the N content of alfalfa. It is suggested that co-inoculation of legumes with antibiotic-producing microorganisms and root-nodule bacteria resistant to those antibiotics is a promising means of promoting nodulation and possibly nitrogen fixation.

Introduction

The rhizosphere contains a large, heterogeneous, and metabolically active community of bacteria. Although organic compounds are excreted by growing roots to sustain the community, competition in this environment must be intense in view of the size, diversity, and biochemical activity of the community. To become a significant member of this community, therefore, an introduced bacterial species must be able to cope effectively with the stress of competition from bacteria that are already well suited to this habitat and which appear to grow quickly. An organism, such as a species of Rhizobium or Bradyrhizobium, that grows more slowly than many indigenous species of the rhizosphere presumably is at a competitive disadvantage and will not develop extensively because of this competition. Such intergeneric interactions have received little attention, although many investiga-

tions have dealt with intraspecific competition among strains of Rhizobium or Bradyrhizobium (Amarger, 1981; Bohlool and Schmidt, 1973).

Intergeneric interactions that deleteriously affect colonization of the legume rhizosphere by rhizobia have been of some interest in the past. For example, the lack of response of subterranean clover to seed inoculation with *Rhizobium trifolii* was attributed to an unspecified type of antagonism that prevented colonization of the root surfaces by *R. trifolii* in the field (Hely *et al.*, 1957). Studies by Harris (1973) of *R. trifolii* grown under artificial conditions indicated that species of Fusarium, Sclerotina, and Hormodendrum decreased nodulation of subterranean clover, and a subsequent investigation by Anderson (1957) demonstrated that several bacteria reduced the number of nodules or prevented nodulation of white clover grown on agar, although the antagonistic organisms did not produce antibiotics acting on *R. trifolii*. Similarly, strains of Azospirillum that did not produce toxins against *R. trifolii* inhibited nodulation of subterranean and white clover (Plazinski and Rolfe, 1985). Further evidence of the significance of indigenous bacteria by addition of antibacterial antibiotics permitted extensive growth of species of Rhizobium and Bradyrhizobium, whereas suppression of eucaryotes resulted in less enhancement (Pena-Cabriales and Alexander, 1983). Similarly, bacteria in the rhizosphere of alfalfa were suppressed and growth of a streptomycin-resistant strain of *R. meliloti* was enhanced as a result of addition of streptomycin to nonsterile soil in which the plants were grown, whereas inoculation of a bacterial mixture into sterile soil inhibited colonization by *R. meliloti* and its nodulation of alfalfa roots (Li and Alexander, 1986).

In view of the likely importance of intergeneric competition in colonization of the legume rhizosphere and nodulation by Rhizobium and Bradyrhizobium, a new approach was attempted to reduce this competition. The approach involved joint inoculation with the root-nodule bacterium and a second species that produced antibiotics that hopefully would suppress species competing with the root-nodule bacteria. To prevent inhibition of the Rhizobium or Bradyrhizobium used in the inoculum, it was first made resistant to the antibiotic.

Materials and methods

Samples of soil were collected from a depth of 0 to 15 cm from Eel silt loam (fine-loamy, mixed, nonacid, mesid family of Aquic Udifluvents). The pH of the soil (soil:water ratio of 1:1) was 6.8. The soil was dried in air and passed through a 2-mm sieve. A solution of K_2HPO_4 was added to the soil before planting to give a final concentration of added P of 500 mg per kg.

Rhizobium meliloti 102F85SE1 is a streptomycin- and erythromycin-resistant mutant derived from *R. meliloti* 102F85, which was originally obtained from Nitragin Co., Milwaukee, WI. *Bradyrhizobium japonicum* 138-SE10 is a streptomycin- and erythromycin-resistant mutant of *B. japonicum* 3I1b138, which was originally obtained from the U.S. Department of Agriculture, Beltsville, MD. The mutants grow in the presence of 1.0 mg of streptomycin and 50 μg of erythromycin per ml. *Streptomyces griseus* ATCC10137, which produces streptomycin, was provided by Tetsuo Ohnuki, Sanraku Inc., Kanagowa, Japan.

Antibiotic-producing bacteria were isolated from Eel silt loam by plating soil dilutions on nutrient agar and picking colonies appearing after 2 days that produced zones of inhibition to nearby colonies on the plate. One isolate is a gram negative, rod-shaped bacterium (1.2 $\times$ 0.6 μm) with one or two polar flagella. It is oxidase positive, catalase negative, liquifies gelatin, and grows on arabinose, glycerol, and glucose. It is designated *Pseudomonas* sp. The second isolate is a gram positive, rod-shaped bacterium that hydrolyzes starch and produces heat-resistant endospores in 10-day-old cultures. It is considered to be a *Bacillus* sp.

The culture medium used for the rhizobia and bradyrhizobia was yeast extract-mannitol agar (Li and Alexander, 1986) amended with 1.0 mg of streptomycin per liter. *S. griseus* was cultured in a medium containing 15 g of malt extract, 5 g of yeast extract, 5 g of soluble starch, 3 g of $CaCO_3$, and 20 g of agar per liter of water. A natural mixture of soil bacteria was grown in a medium containing 1.0 g of glucose, 0.5 g of K_2HPO_4, 100 ml of soil extract, and 15 g of agar per liter of water; the pH was adjusted to 7.4 before adding the agar. Soil extract was prepared by autoclaving 1000 g of Eel silt loam

with 1000 ml of tap water for 30 min. $CaCO_3$ was added to the mixture, which was repeatedly passed through Whatman no. 1 filter paper to obtain a clear solution.

To test for their antibiotic-producing activity, *Pseudomonas* sp. and *Bacillus* sp. were grown in nutrient broth for 24 h. The cells were collected by centrifugation and washed twice with sterile water, and then the cell suspensions were diluted in sterile water and added to 20 g of sterile or nonsterile Eel silt loam to give 23% moisture (wt/wt). Phosphate was added to give 500 mg of P per kg of soil. The soil then was incubated at 27°C. After 3 or 7 days, the soil was mixed well with 25 ml of sterile water, and the soil suspension was centrifuged at 7,700 × g for 20 min. A drop of the resulting supernatant fluid was placed on sterile paper discs, and the discs were placed on the surface of soil-extract agar (1.5% agar in soil extract) containing a lawn of soil bacteria. Lawns were prepared by spreading a bacterial suspension evenly over the surface of soil-extract agar. The 0.9 to 1.1 mm zone of clearing around the discs was indicative of antibiotic activity.

To obtain mutants resistant to the antibiotics produced by *Bacillus* sp. or *Pseudomonas* sp., these bacteria were grown for 2 days at 30°C in nutrient broth on a shaker operating at 120 rpm. The cells were then removed by centrifugation at 7,700 g for 10 min, and the supernatant fluid was added to yeast extract-mannitol broth in ratios of 1:3, 1:1 or 9:1. *R. meliloti* or *B. japonicum* was inoculated into the 1:3 mixture, and the cultures were incubated at 30°C on the shaker. When growth was evident, the cultures were plated on an agar medium containing the 1:3 mixture. Single colonies that appeared were inoculated into broth with a 1:1 ratio of the two components, and the procedure was repeated using media with the 1:1 and 9:1 ratio of the two components. A similar procedure was used to obtain mutants resistant to the antibiotic of *S. griseus*, which was grown in starch-malt extract-yeast extract broth. All studies were done with these mutants, and their abundance was determined on yeast extract/mannitol agar supplemented with 1.0 mg of streptomycin and 50 μg of erythromycin per ml. The plates were incubated at 30°C for 5 to 7 days.

Seeds (20 g) of alfalfa (*Medicago sativa* L, variety Oneida VR) or soybean (*Glycine max* (L) Merrill, variety Evans) were inoculated with 0.4 ml of 5-day-old cultures of *R. meliloti* or *B. japonicum* containing 2×10^7 cells per ml, and the seeds were then dried at room temperature and mixed well with 2.0 ml of sterile 40% (wt/vol) gum arabic. Seeds (20 g) were inoculated with 0.4 ml of an 8-day-old culture of *S. griseus* contaioning 10^4 cells per ml, they were amended with 0.2 g of ground chitin that had been passed through a 40-mesh sieve, or they received both *S. griseus* and chitin. The seeds were dried at room temperature or under forced ventilation before planting.

The plants were grown in 15-cm diameter pots containing 2 kg of soil. The soil was brought to a moisture level of 25% with sterile water, and water was added every other day to maintain the moisture level. The seeds were germinated on 1% water agar, and after 2 days, the seedlings were planted in the pots, which were then placed in a growth chamber at 27°C under a light intensity of 250 μmol photon/m^2 per sec.

Rhizosphere counts were performed by removing the plants from the soil and discarding the soil that was removed by gently shaking the roots. The remaining plant material was shaken vigorously by hand, and bacterial counts were made of the resulting suspension.

The plants were harvested, washed with water to remove soil particles, and dried at 105°C for dry-weight determinations. Soybean pods were also dried at 105°C. For N analysis, the plants were dried at room temperature and ground in a Wiley mill, the ground samples were dried overnight at 70°C, and a Perkin-Elmer model 240C elemental analyzer equipped with autosampler was used for the determination.

In studies in which the soil was inoculated, the seeds were planted after the inoculum was added to the soil, and the soil was adjusted to a moisture content of 25% (wt/wt).

Ground chitin was sterilized by autoclaving for 1 h. The surfaces of seeds were sterilized by suspending them in 70% ethanol for 1 min, washing them once with sterile distilled water, soaking them for 5 min in 50% Na hypochlorite, and then washing them 7 to 10 times with sterile water. When sterile soil was used, it was amended with K_2HPO_4 to give 325 mg of P per kg of soil, added to 30-mm diameter test tubes to give 24.6 g wet weight of soil at 23% moisture, and autoclaved for

1 h. The tubes were then incubated at 30°C for 24 h and subsequently autoclaved for 1 h. The pH was 6.8. No growth appeared when samples were transferred to nutrient broth.

To obtain inocula of known cell densities, bacterial suspensions used for counting were stored at 4°C until the cell numbers were determined, and appropriate dilutions were then prepared to obtain the desired density.

Results

Soil was inoculated with *R. meliloti* at an initial density of 2.8×10^5 cells per gram and *Pseudomonas* sp. at initial densities of 3.7×10^5, 3.7×10^6, and 3.7×10^7 per g, and alfalfa was grown in the soil. The density of *R. meliloti* in the rhizosphere increased with time as the plants grew (Table 1). Addition of *Pseudomonas* sp. at initial population sizes of 3.7×10^6 and 3.7×10^7 per g of soil resulted in greater numbers of *R. meliloti* at each sampling date, although the counts were statistically higher at only four of the five sampling dates. A statistically significant response to the smallest *Pseudomonas* sp. inoculum size was observed at only two sampling times, and these were both in the first 7 days.

A series of identical experiments was conducted with the *R. meliloti* inoculum increased to 2.8×10^6 cells per g of soil. In this instance, however, the tests with each *Pseudomonas* sp. inoculum size was conducted at different times. The beneficial effect of the antagonist in this study was restricted to the first week (Table 2). *R. meliloti* was most markedly stimulated by the largest inoculum of *Pseudomonas* sp. Although the numbers of *R. meliloti* were greater at four of the five sampling times when *Pseudomonas* sp. was added to an initial density of 3.7×10^6/g, only one of these apparent responses was statistically significant. However, *Pseudomonas* sp. at an inoculum size of 3.7×10^5/g had a stimulatory effect at two of the three sampling times in the first week.

Table 1. The influence of inoculation with *Pseudomonas* sp. on growth of *R. meliloti* in the rhizosphere of alfalfa

Days	No. of *R. meliloti* × 10^6/g rhizosphere soil			
	0[a]	3.7×10^5[a]	3.7×10^6[a]	3.7×10^7[a]
3	0.71 A[b]	1.58 B	1.57 B	1.02 B
5	3.54 A	2.30 A	16.4 B	11.5 B
7	8.41 A	12.4 B	12.8 B	14.0 B
14	16.8 A	23.6 A	29.6 A	30.5 A
21	17.7 A	27.9 AB	62.7 B	36.4 B

[a]No. *Pseudomonas* sp. added per g soil.
[b]In this and succeeding tables, values in a row followed by different letters are significantly different ($P < 0.05$).

Table 2. The effect of *Pseudomonas* sp. on the population of *R. meliloti* in the alfalfa rhizosphere

Pseudomonas sp. added per g of soil	Days	No. of *R. meliloti* × 10^6/g of rhizosphere soil	
		Without *Pseudomonas* sp.	With *Pseudomonas* sp.
3.7×10^5	3	6.13	13.2*[a]
	5	5.57	10.2*
	7	34.7	32.1
	14	56.5	39.9
	21	60.6	80.7
3.7×10^6	1	31.8	21.2
	2	24.8	44.3
	3	29.3	55.9
	4	47.9	81.1
	7	60.3	118*
	16	52.1	71.7
	21	39.6	63.5
3.7×10^7	2	17.5	110**
	4	37.5	122**
	6	61.5	121*
	15	44.5	75.5
	21	33.6	69.7

[a]*,**Significantly different ($P < 0.05$ and 0.01, respectively) from values for plants grown in soil without *Pseudomonas* sp. Other differences were not statistically significant.

A study was conducted to determine whether *Pseudomonas* sp. would also enhance nodulation induced by *R. meliloti* and alfalfa growth. Rhizosphere colonization by *R. meliloti* was again measured. The initial *R. meliloti* density was 2.8×10^5/g of soil, and *Pseudomonas* sp. was added to give 3.7×10^5, 3.7×10^6, and 3.7×10^7/g of soil. In this instance, the largest *Pseudomonas* sp. inoculum increased *R. meliloti* numbers up to 15 days (Table 3). The 20-day *R. meliloti* count was more than twice that in the absence of the pseudomonad, but the difference was not statistically significant. A beneficial effect in the first 15 days was also noted when *Pseudomonas* sp. was added at an initial density of 3.7×10^6/g but not at the lower inoculum level. Measurements of nodule numbers reflected the be-

Table 3. Effect of *Pseudomonas* sp. on *R. meliloti* colonization of the alfalfa rhizosphere and on alfalfa nodulation

Measurements	Days	0[a]	3.7×10^{5}[a]	3.7×10^{6}[a]	3.7×10^{7}[a]
No. of *R. meliloti* $\times 10^{6}$/g rhizosphere soil	2	7.80 A	9.66 A	3.08 A	8.59 A
	4	23.5 A	59.0 A	149 B	96.4 B
	7	61.3 A	60.3 A	117 B	121 B
	15	6.84 A	9.57 A	15.9 B	37.1 B
	20	32.9 A	51.6 A	76.7 A	75.6 A
	40	14.4 A	63.3 A	36.4 A	9.86 B
	60	31.5 A	63.0 A	62.9 A	22.5 A
No. of nodules per 12 plants	60	96 A	99 A	142 B	142 B

[a]No. of *Pseudomonas* sp. added per g of soil.

neficial influence of the two largest pseudomonad inocula, the numbers at 60 days being greater as a result of the antagonist's presence. On the other hand, determinations of plant weight showed no enhancement. The dry weights at 20, 40, and 60 days averaged for all treatments were 13.3, 27.7 and 87.0 mg per three plants, respectively.

A similar experiment was conducted with *Bacillus* sp. The root-nodule bacterium in this instance was *B. japonicum*, and it was inoculated into soil to give 2.8×10^{5} cells per g. *Bacillus* sp. was added at three cell densities into soil in which soybeans were grown. The antibiotic-producing isolate increased the *B. japonicum* numbers as early as 3 days, and each inoculum size of the antibiotic producer had a stimulatory effect (Table 4). The response was still evident at 28 days although, surprisingly, not at 16 days. The numbers of nodules on the 28-day old plants were 28, 45, 52, and 12 per six plants for soybeans grown in soil inoculated with 0, 3.7×10^{5}, 3.7×10^{6}, and 3.7×10^{7} *Bacillus* sp. per g; the values for the two lower inoculum sizes were significantly greater and the value for the largest inoculum size was significantly less than for plants grown in soil not receiving *Bacillus* sp. ($P < 0.05$). The low nodule number may have resulted from the small *B. japonicum* inoculum. The average weight of the 28-day-old plants was 154 mg, and treatment with the antagonist had no effect on plant yield.

Table 4. Effect of *Bacillus* sp. on *B. japonicum* in the soybean rhizosphere

Days	No. of *B. japonicum* $\times 10^{6}$/g rhizosphere soil			
	0[a]	3.7×10^{5}[a]	3.7×10^{6}[a]	3.7×10^{7}[a]
3	6.85 A	44.2 B	31.2 B	131 C
5	10.6 A	180 B	94.3 B	168 B
7	28.6 A	153 B	98.9 B	95.7 B
16	64.5 A	77.4 A	74.0 A	43.9 A
28	79.0 A	126 B	131 B	114 B

[a]No. of *Bacillus* sp. added per g of soil.

The effect of seed inoculation with *Bacillus* sp. was also evaluated. The seeds also were inoculated with *B. japonicum* for soybeans and *R. meliloti* for alfalfa (8×10^{6} cells per 20 g of seeds), and they were coated with gum arabic. The whole alfalfa root system was examined, but only the 0–2 or 0–3 cm segments of soybean roots (distances are measured from the point on the plant where it emerged from the soil) were excised and examined for *B. japonicum* densities. At least 4 replicates of each plant species were evaluated. The test of soybeans was part of a separate study of the movement of *B. japonicum* on the root system, the details of which will be published separately.

Inoculation of seeds with *Bacillus* sp. enhanced root colonization. The stimulation of *B. japonicum* was evident at 6 and 10 days, but the stimulation of *R. meliloti* was only observed at 3 days (Table 5). No beneficial influence was noted thereafter. However, even though the stimulation of the root-nodule bacteria was limited to the early period of plant growth, nodulation of both species was promoted.

A study was conducted in which soil was mixed well with 0.0, 0.25, or 1.0% chitin and then inoculated with 10^{4} *S. griseus* cells per g of soil. The soil was inoculated with 9.6×10^{5} *R. meiloti* cells per g, and alfalfa was sown in the soil. *S. griseus* alone had no effect on *R. meliloti* in the alfalfa rhizosphere, except for the slight stimulation at 26 days (Table 6). On the other hand, the treatment containing *S. griseus* and chitin caused a marked rise in the numbers of *R. meliloti* at 10, 19, and 26 days.

Table 5. Effect of seed inoculation with *Bacillus* sp. on colonization and nodulation of soybeans by *B. japonicum* and alfalfa by *R. meliloti*

Root-nodule bacterium	Days	Without *Bacillus* sp.	With *Bacillus* sp.
	No. of root-nodule bacteria × 10^6/g rhizosphere soil		
B. japonicum	6	2.35[a]	10.8[a]*
	10	0.237[a]	14.2[a]*
	42	111[b]	258[b]
R. meliloti	3	14.8	42.7*
	5	416	141
	7	27.6	47.2
	19	106	131
	No. of nodules[c]		
B. japonicum	42	22	34*
R. meliloti	27	31	53*

*Significantly different from plants not inoculated with *Bacillus* sp. ($P < 0.05$).
[a]On 0–3 cm root segment.
[b]On 0–2 cm root segment.
[c]No. per soybean plant or per 12 alfalfa plants.

The stimulation was evident with both chitin levels. *S. griseus* alone did not result in an increase in nodule number at 26 days, but the mixture of *S. griseus* with the lower chitin level did have a beneficial effect. It is interesting that the higher chitin level was deleterious.

Alfalfa seeds were coated with gum arabic and *R. meliloti* and then with (i) *S. griseus*, (ii) chitin, (iii) *S. griseus* and chitin, or (iv) nothing. Inoculation of the seeds with *S. griseus* alone had no effect on *R. meliloti* (Table 7). Treatment of the seeds with chitin reduced the *R. meliloti* counts at two sampling dates but produced no statistically significant change at the other four sampling periods. On the other hand, the application of chitin plus *S. griseus* led to a marked rise in the abundance of *R. meliloti* in the rhizosphere of 3- and 5-day old plants, but the effect disappeared with time. *S. griseus* alone increased nodulation at some sampling dates and caused a small increase in dry weight and N content of 70-day-old plants. Chitin alone had no effect on plant yield or N content, and it affected nodule number only at 34 days. On the other hand, seed treatment with both chitin and *S. griseus* led to a marked increase in nodule number, plant weight and N content at each sampling date.

To determine whether chitin had a direct influence on *R. meliloti*, the bacterium was inoculated into sterile soil to give 1.1×10^6 cells per g. The soil was then amended with 0.0 or 0.1% sterile chitin. Surface-sterilized seeds of alfalfa were planted in the soil. Counts at 3, 6, 12, 18, and 24 days showed that chitin had no direct effect on *R. meliloti* in the alfalfa rhizosphere. An identical experiment was conducted but without the plants. Counts of the soil at 3, 6, 12, 18, and 24 days showed that chitin did not enhance *R. meliloti* growth following its inoculation into sterile soil.

Soybean seeds were coated with gum arabic and an inoculum of *B. japonicum*. The seeds were also coated with (i) *S. griseus*, (ii) chitin, (iii) *S. griseus* plus chitin, or (iv) nothing. Chitin alone and *S. griseus* alone did not affect colonization of the soybean rhizosphere by *B. japonicum*, but a mixture of the two enhanced its numbers in the rhizosphere of 6- and 14- but not 44-day-old plants (Table 8). Nodule number and root weight were also enhanced by *S. griseus* plus chitin, although lesser effects were observed with the individual amendments.

Soybean plants were grown to maturity to determine the effect of *Bacilllus* sp. and chitin plus *S. griseus* on nodulation and growth. Seeds received

Table 6. Effect of chitin and *S. griseus* on *R. meliloti* and alfalfa nodulation and growth

Measurements	Days	Soil treatment			
		None	*S. griseus*	*S. griseus*, 0.25 chitin	*S. griseus*, 1% chitin
No. of *R. meliloti* × 10^6/g rhizosphere soil	3	2.64 A	6.21 A	6.14 A	7.13 A
	10	181 A	260 A	507 B	982 C
	19	98.1 A	20.1 A	251 B	429 B
	26	85.4 A	136 B	359 B	228 B
No. of nodules (per 12 plants)	26	39 A	49 A	60 B	2 C

Table 7. Effect of chitin and *S. griseus* addition to alfalfa seeds on *R. meliloti* numbers and alfalfa nodulation and growth

Measurements	Days	Treatments			
		None	*S. griseus*	Chitin	*S. griseus*, chitin
No. of *R. meliloti*	3	79.0 A	25.5 A	20.5 A	635 B
$\times 10^6$/g	5	25.2 A	13.4 A	3.04 C	143 B
rhizosphere soil	7	23.1 A	15.8 A	14.9A	12.3 A
	19	62.8 A	58.2 A	22.9 A	34.5 A
	34	192 A	157 A	65.4 B	221 A
No. of nodules	27	28 A	23 A	26 A	53 B
per 12 plants	34	38 A	57 B	51 B	80 C
	70	92 A	145 B	87 A	148 B
Dry weight	34	25.9 A	25.1 A	24.2 A	34.5 B
(mg/3 plants)	70	242 A	254B	248 A	270 C
N content (mg/3 plants)	70	9.41A	10.6 B	9.97 AB	11.6 C

gum arabic and an inoculum of *B. japonicum*, and they were treated with (i) *Bacillus* sp., (ii) *S. griseus* plus chitin, or (iii) nothing. Some seeds were left uninoculated. The additions were made to give 4.2×10^7 *B. japonicum* cells, 1.3×10^8 *Bacillus* sp. cells, 9.0×10^5 *S. griseus* cells, or 0.80 g of ground chitin per 20 g seeds. *Bacillus* sp. and *S. griseus* with chitin enhanced nodulation by *B. japonicum*, the fresh weights of seeds, and the dry weights of whole plants (Table 9). *Bacillus* sp. also increased the number and dry weights of the pods. *Bacillus* sp. had a more pronounced influence than *S. griseus*.

Discussion

The present study suggests a new means for enhancing colonization of the legume rhizosphere and for stimulating nodulation by species of Rhizobium and Bradyrhizobium. The assumptions underlying this approach are that these bacteria are being suppressed by competing species of bacteria and that the latter can be controlled by species producing toxins active against the competitors but with little or no effect on the root-nodule bacteria or the host plant. However, to be effective, that antibiotic-producing organism must be able to multiply and produce the inhibitor in the rhizosphere, and it must be present at the same sites on the root surface that are occupied by the rhizobia or bradyrhizobia. If multiplication and antibiotic production does not occur or if the second species in the inoculant does not exist at sites where competitive stress occurs, the co-inoculation approach will fail.

Nevertheless, these data show that success has been achieved with three different antibiotic producers. *S. griseus* is the source of streptomycin in commercial practice, antibiotic-forming isolates of *Pseudomonas fluorescens* have been used for the

Table 8. Effect of chitin and *S. griseus* on soybean growth, nodulation, and rhizosphere colonization

Measurements	Days	Treatments			
		None	*S. griseus*	Chitin	*S. griseus*, chitin
No. of *B. japonicum*	6	0.892 A	1.09 A	0.914 A	1.82 B
$\times 10^6$/g	14	3.30 A	2.61 A	1.06 A	10.4 B
rhizosphere soil	44	20.8 A	21.0 A	10.9 A	11.6 A
No. of nodules per plant	44	21 A	33 B	23 A	44 C
Dry weight of roots (mg)	44	530 A	668 B	612 B	731 C

Table 9. Effect of the addition to seeds of *Bacillus* sp. or chitin and *S. griseus* on soybean nodulation and growth after 76 days

Measurements	Treatments			
	None	*B. japonicum*	*B. japonicum*, *Bacillus* sp.	*B. japonicum*, *S. griseus*, chitin
No. of nodules per 6 plants	46 A	134 B	210 C	193 C
No. of pods per plant	9 A	7 A	13 B	9 A
Fresh seed weight (g/plant)	1.75 A	2.24 B	2.60 D	2.42 C
Dry weight of pods (g/plant)	1.06 A	1.28 B	1.45 C	1.31 B
Dry weight of entire plant (g/plant)	5.94 A	5.44 A	7.70 C	6.21 B

biological control of *Rhizoctonia solani* (Howell and Stipanovic, 1979), and antibiotic-forming strains of *Bacillus subtilis* protect carnations against *Fusarium oxysporum* f. sp. *dianthi* (Filippi *et al.*, 1987). It is not certain, however, that the beneficial effect from co-inoculation of antibiotic producers with *R. meliloti* and *B. japonicum* results from the formation in the rhizosphere of the toxin that is produced in culture media. An analogous approach has been suggested to minimize intraspecific competition of *R. trifolii* by inoculation with bacteriocin-producing and bacteriocin-resistant strains of this species (Hodgson *et al.*, 1985). Such approaches are logical extensions of other studies that demonstrate that pesticides may stimulate the populations, activities, or both of *Rhizobium*, *Bradyrhizobium*, and free-living nitrogen-fixing heterotrophs and cyanobacteria by suppressing organisms that prey on or compete with the nitrogen fixers (Alexander, 1985; Hossain and Alexander, 1984a, 1984b).

It is evident that the beneficial effect is a function of size of the inoculum of the antibiotic producer. This is not surprising because a sufficient density of that organism must be present to generate enough of the toxin to suppress competing bacteria. The data also show that colonization is benefitted only during the early periods of plant growth. The reason for the limited time in which colonization is promoted is uncertain, but it could result from the preferential growth of indigenous rhizosphere bacteria naturally resistant to the antibiotic, the proliferation of microorganisms able to degrade the chemical, or the disappearance of conditions that are favorable to antibiotic synthesis so that *R. meliloti* or *B. japonicum* no longer has a selective advantage. Nevertheless, nodulation and nitrogen fixation still benefitted from the co-inoculation, so that the preferential stimulation of the root-nodule bacteria during the early phases of legume development presumably allowed for the beneficial influence to be maintained.

Chitin amendments have long been known as a means of selectively stimulating the growth of actinomycetes and for enhancing the biological control of certain plant pathogens (Mitchell and Alexander, 1962). More recently, Polyanskaya *et al.* (1985) and Limar *et al.* (1984) reported that amendment of soil with 1% chitin promotes the growth both of an inoculum of *Streptomyces olivocinereus*, which forms the antibiotic heliomycin in culture media, and of *Rhizobium leguminosarum*. Heliomycin was reported to be active against gram-positive bacteria. However, the stimulation of *R. leguminosarum* was attributed to its use of products of the enzymatic hydrolysis of chitin and not to the antibiotic (Polyanskaya *et al.* 1985). Soil treatment with quantities of chitin as large as 1% (wt/wt) is not economically feasible, and mixing chitin with soil in the field is difficult; moreover, chitin added to soil at such large concentrations was found in the present study to suppress nodulation. On the other hand, only small quantities of chitin are applied to seeds as a coating and the procedure is simple so that co-inoculation of chitin-coated seeds with *S. griseus* is a feasible means of stimulating rhizobia or bradyrhizobia.

The data thus suggest that co-inoculation of

root-nodule bacteria with antibiotic-producing microorganisms is a promising technique for stimulating nodulation and nitrogen fixation by the legume-bacterial symbiosis. Better strains of antibiotic producers can undoubtedly be found, but additional tests are still warranted to determine whether these first observations will be supported by subsequent experimentation, especially under natural conditions in the field.

References

Alexander M 1985 Enhancing nitrogen fixation by use of pesticides: a review. Adv. Agron. 38, 267–282.

Amarger N 1981 Selection of *Rhizobium* strains on their competitive ability for nodulation. Soil Biol. Biochem. 13, 481–486.

Anderson K J 1957 The effect of soil micro-organisms on the plant-rhizobia association. Phyton (Rev. Int. Bot. Exp.) 8, 59–73.

Bohlool B B and Schmidt E L 1973 Persistence and competition aspects of *Rhizobium japonicum* observed in soil by immunofluorescence microscopy. Soil Sci. Soc. Am. Proc. 37, 561–564.

Filippi C, Bagnoli G, Volterrani M and Picci P 1987 Antagonistic effects of soil bacteria on *Fusarium oxysporum* f. sp. *dianthi* (Prill and Del) Snyd. and Hans. Plant and Soil 98, 161–167.

Harris J R 1953 Influence of rhizosphere micro-organisms on the virulence of *Rhizobium trifolii*. Nature (London) 172, 507–508.

Hely F W, Bergerson F J and Brockwell J 1957 Microbial antagonism in the rhizosphere as a factor in the failure of inoculation of subterranean clover. Aust. J. Agric. Res. 8, 24–44.

Hodgson A L M, Roberts W P and Waid J S 1985 Regulated nodulation of *Trifolium subterraneum* inoculated with bacteriocin-producing strains of *Rhizobium trifolii*. Soil Biol. Biochem. 17, 475–478.

Hossain A K M and Alexander M 1984a Enhancing soybean rhizosphere colonization by *Rhizobium japonicum*. Appl. Environ. Microbiol. 48, 468–472.

Hossain A K M and Alexander M 1984b Enhancing growth and nitrogen uptake by soybeans using pesticides. Plant and Soil 81, 133–141.

Howell C R and Stipanovic R D 1979 Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69, 480–482.

Li D-M and Alexander M 1986 Bacterial growth rates and competition affect nodulation and root colonization by *Rhizobium meliloti*. Appl. Environ. Microbiol. 52, 807–811.

Limar T E, Polyanskaya L M, Kozhevin P A and Zvyagintsev D G 1984 Measures for increasing the numbers of nodule bacteria in soil (*In Russian*). Mikrobiologiya 53, 830–832.

Mitchell R and Alexander M 1962 Microbiological processes associated with the use of chitin for biological control. Soil Sci. Soc. Am. Proc. 26, 556–558.

Pena-Cabriales J J and Alexander M 1983 Growth of Rhizobium in soil amended with organic matter. Soil Sci. Soc. Am. J. 47, 241–245.

Plazinski J and Rolfe B G 1985 Influence of Azospirillum strains on the nodulation of clovers by Rhizobium strains. Appl. Environ. Microbiol. 49, 984–989.

Polyanskaya L M, Kozhevin P A and Zvyagintsev D G 1985 Stimulation and elimination of nodule bacteria in soil after the introduction of an actinomycete and chitin (*In Russian*). Mikrobiologiya 53, 1012–1015.