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Effects of Rhizobacteria on Soybean Cyst Nematode, *Heterodera glycines*[1]

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Abstract: Rhizobacteria were isolated from the rhizoplane and rhizosphere of soybean plants from fields in Arkansas and tested for their effect on numbers of soybean cyst nematode (*Heterodera glycines*). In initial greenhouse tests in heat-treated silt loam soil, 138 of the 201 bacterial isolates tested had no influence on numbers of cysts and eggs + second-stage juveniles (J2) of *H. glycines,* 36 reduced (suppressive isolates) and 27 increased (enhancing isolates) numbers of cysts and (or) eggs + J2 when compared to the controls ($P \leq 0.05$). When 20 suppressive and five enhancing isolates were retested in the same soil, the results were highly variable and inconclusive. The 25 isolates were then evaluated in vitro for their effects on eggs and J2 of *H. glycines.* No clear relationship was detected between the inhibition of egg hatch or immobilization of J2 in vitro and antagonistic activity toward nematodes in vivo. Amendment of the soil with 0.1% (w/w) peptone or casein hydrolysate did not improve the effects of suppressive isolates on numbers of *H. glycines.* Nineteen of the 25 isolates were identified based on analysis of fatty acid methyl esters, and they are in 11 different genera.

Key words: biological control, *Glycine max, Heterodera glycines,* rhizobacteria, soybean, soybean cyst nematode.

Soybean cyst nematode (*Heterodera glycines* Ichinohe), a yield-limiting pest of soybean (*Glycine max* (L.) Merr.), is widespread in Eastern Asia and North and South America and continues to spread throughout the soybean production areas of the world (Noel, 1992). In the southern United States, total yield losses caused by *H. glycines* were greater than those caused by any other disease during 1974 to 1996 (Pratt and Wrather, 1998; Wrather et al., 1995). Traditionally, resistant cultivars, crop rotation, and nematicides have been used for management of *H. glycines.* However, these practices have limitations and give insufficient levels of control (Riggs and Schuster, 1998). This situation has stimulated research in the direction of new management strategies, such as biological control, that can complement or replace existing practices. Parasites and predators of *H. glycines* have been the primary focus of research for biological control of *H. glycines.* However, field applications of these antagonists often have failed to suppress nematode populations because of their inability to compete with the indigenous microorganisms (Kim and Riggs, 1992; Stirling, 1991).

Recently, rhizobacteria, which are bacteria that colonize roots in the presence of the indigenous soil microflora (Schroth and Hancock, 1982), have shown promise as biocontrol agents of nematodes. Genera of rhizobacteria that reduce nematode populations include *Agrobacterium, Alcaligenes, Bacillus, Clostridium, Desulfovibrio, Pseudomonas, Serratia,* and *Streptomyces,* and the nematode targets are species in diverse genera including *Belanolaimus, Caenorhabditis, Criconemella, Globodera, Heterodera, Meloidogyne, Panagrellus, Pratylenchus, Rotylenchulus,* and *Tylenchorhynchus* (Siddiqui and Mahmood, 1999). Although a variety of nematodes have been studied, most research has focused on biocontrol of *M. incognita* and *H. schachtii* by rhizobacteria. In the only study examining the effect of rhizobacteria on *H. glycines,* Kloepper et al. (1992) found that some *Bacillus* and *Pseudomonas* species reduced cyst numbers.

Little is known about the mechanisms by which rhizobacteria suppress plant-parasitic nematode populations. Rhizobacteria may interfere with the host-finding process by receptor blockage on roots and (or) modification of root exudates of the host plant, thus hindering the attraction, hatching, or penetration behavior of nematodes (Sikora and Hoffmann-Hergarten, 1993). In addition,

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rhizobacteria may produce specific enzymes or metabolites that are nematicidal or reduce egg hatch (Oka et al., 1993). Recently, rhizobacteria, such as *A. radiobacter* and *B. sphaericus,* were reported to induce systemic resistance against *G. pallida* in potato (Hasky-Gunther et al., 1998).

The primary objectives of this research were to test the effect of bacteria isolated from the rhizosphere and rhizoplane of soybean plants on numbers of cysts and eggs + second-stage juveniles (J2) of *H. glycines* in soil and identify bacterial isolates affecting nematode numbers. To determine whether toxins were involved in nematode suppression, the effects of cell suspensions of the bacterial isolates and their cell-free culture filtrates on hatch of eggs and viability of J2 were investigated. In addition, because organic matter may help the establishment of a biocontrol agent in soil (Kerry, 1988), organic amendments were tested to determine if they improved the effectiveness of the bacteria for control of *H. glycines.*

Materials and Methods

Bacterial isolation and inoculum: Soybean roots with adhering soil were randomly collected during the 1996 growing season from soybean fields (10 to 15 plants per field) infested with *H. glycines* in Faulkner, Crawford, Conway, Lee, Mississippi, and St. Francis counties in Arkansas. Bacteria from the rhizoplane and rhizosphere of soybean were isolated following the method described by Wollum (1982) except that the diluent was buffered water prepared by the method of Greenberg et al. (1992). Tryptic soy agar (TSA, pH 7.3 ± 0.2), *Pseudomonas* agar F (PAF, pH 7.0 ± 0.2) (Difco Laboratories, Detroit, MI), and starch casein medium (pH 7.0) (Williams and Wellington, 1982) were used for the isolations. Bacterial colonies, from each sample, representing distinct cultural types, morphology, and color, were purified and stored in 1-ml sterile tryptic soy broth (TSB) (Sigma Chemical Co., St. Louis, MO) or liquid PAF (PAF without agar) with 20% sterile glycerol in cryogenic vials (Nalgene Company, Rochester, NY) at −76 °C.

To prepare bacterial inocula for experiments, bacteria were grown on TSA or PAF overnight at 30 °C before cells were swabbed from the agar surface with sterile cotton-tipped applicators (Hardwood Products Co., Guilford, ME) and placed in sterile deionized water. The concentration of a bacterial suspension was determined at ×1,280 magnification with a hemocytometer and diluted to 10^9 CFU per ml.

Nematode inoculum: Heterodera glycines race 3, used throughout this research, was cultured on susceptible soybean cv. Lee 74 in a greenhouse. Eggs and J2 of *H. glycines* suspended in water were poured into the soil surrounding the roots. After 5 weeks, soil was washed from the roots, roots were rubbed to dislodge females and cysts (hereafter referred to as cysts), and the soil-cyst-water mixture was stirred and poured through nested 850-µm-pore and 250-µm-pore sieves. Cysts were collected from the 250-µm-pore sieve and separated from soil, roots, and debris by sugar flotation (Southey, 1986). Cysts were crushed with a glass tissue grinder to release the eggs and J2, which were then collected on a 25-µm-pore sieve nested under a 75-µm-pore sieve. After thoroughly rinsing the eggs and J2 on the 25-µm-pore sieve with tap water, they were washed into a glass beaker (hereafter referred to as egg suspension). To obtain a large number of J2, about 10 to 20 ml of egg suspension was poured on a moistened Whatman No. 1 filter paper within a Baermann funnel. To stimulate egg hatch, 16 mM $ZnSO_4.7H_2O$ in water was added to the funnel until it just covered the lower surface of the filter paper (Tefft and Bone, 1984). Hatched J2 were collected daily. Freshly obtained egg or J2 suspensions were used in subsequent experiments.

Greenhouse screening tests: Soybean cv. Hutcheson seeds were germinated in vermiculite. Roots of 7-day-old seedlings were washed free of vermiculite in running tap water, rinsed three times in sterile deionized water, and immersed in a bacterial suspension or sterile water (control) for 15 minutes. The seedlings were then planted individually in 7-cm-diam. clay pots with heat-

treated (3 hours at 105 °C) silt loam soil (pH 7.6, 0.5% organic matter, 42.1% sand, 54.9% silt, and 3% clay) from the Arkansas River Valley. An additional 10-ml aliquot of the bacterial suspension was added to the soil around the roots. Treatments were replicated five times and were completely randomized on a greenhouse bench maintained at 22 to 32 °C. After 1 week, two small holes were made in the soil adjacent to the base of the seedling with sterile pipet tips, and 1,000 eggs of *H. glycines,* which had been washed three times in sterile deionized water, were pipeted into each hole. The holes were covered with a thin layer of the heat-treated soil. Four weeks later, cysts were extracted as described previously and counted. Cysts were then crushed, and eggs and J2 were collected and counted. The percentage difference in the number of cysts or eggs + J2 relative to the control was calculated as (treatment mean – control mean) × 100/control mean. A positive value represented an increase and a negative value a reduction in cyst or eggs + J2 numbers.

Twenty bacterial isolates that resulted in ≥50% reduction of cyst and (or) eggs + J2 numbers (suppressive isolates) and five bacterial isolates that resulted in >100% increase in both cyst and eggs + J2 numbers (enhancing isolates) compared to the water controls were retested twice for their effect on *H. glycines* numbers in the heat-treated silt loam soil. The same procedure as described above was used except that treatments were replicated 10 times in one test and 12 times in the other.

Effects of bacterial cell suspensions on egg hatch and J2 viability: Bacterial suspensions containing 10^9 CFU per ml of the 20 suppressive and five enhancing isolates were prepared as above. Nematode egg or J2 suspensions were centrifuged at 3,000*g* for 15 minutes, and the supernatant was carefully removed with a pipet. The egg or J2 pellet was disinfected with a solution containing 5 g/liter chlorhexidine and 100 µg/ml rifampicin for 30 minutes, washed three times with sterile deionized water by centrifugation and pipeting, and diluted to 1,000–2,000 eggs or 150–350 J2 per ml of sterile deionized water with 0.05% (v/v) Triton X-100 solution. The antibiotic solution, in previous tests, had no effect on egg hatch or J2 viability. Triton X-100, which was used as a wetting agent to prevent eggs and J2 from adhering to the bottoms of petri dishes, had no effect on egg hatch or J2 viability at the concentration used (data not shown). To determine effects of bacteria on egg hatch or J2 viability, a 1-ml egg or J2 suspension and a 5-ml bacterial suspension were pipeted into a 60 × 15-mm sterile disposable plastic petri dish and incubated in the dark at 24 °C. Evaporation from dishes was limited by placing them in a plastic box that was loosely covered. Each treatment was replicated 10 times. Sterile water served as a control. After incubation of J2 for 48 hours, the first 100 J2 in each of five dishes were checked for mobility and mortality. Juveniles were considered immobile if they were straight and not moving and dead if no contents were immediately extruded when the body was cut with a knife. The J2 in the remaining five dishes were poured into soil around roots of 2-week-old seedlings of soybean cv. Hutcheson planted in heat-treated silt loam soil in 7-cm-diam. clay pots. Pots were completely randomized on a greenhouse bench maintained at 22 to 32 °C. After 4 weeks the cysts were extracted and counted. Eggs in five of the dishes were incubated for 4 days and then poured into soil as with J2. Four weeks later the cysts were extracted and counted. Eggs in the other five dishes were incubated for 10 days before hatch rate was determined on the first 100 eggs plus J2 observed. At the end of incubation, a loop of the solution was streaked on TSA or PAF to determine whether the bacterial isolates were recoverable. All the tests were repeated once.

Effects of culture filtrates on egg hatch and J2 viability: Culture filtrates of the bacterial isolates from TSB and soybean root exudate were tested for presence of toxins to eggs and J2 of *H. glycines.* To obtain cell-free TSB-culture filtrates of the 20 suppressive and five enhancing bacterial isolates, a loop of a fresh bacterial culture was transferred into 100 ml TSB in a 250-ml flask, and the flask was incubated at 28 °C on a rotary shaker

(150 rpm) for 24 hours. The broth culture was centrifuged at 4 °C at 10,242*g* for 15 minutes, and the supernatant was sterilized by filtration through a 0.2-µm-pore cellulose nitrate membrane (Nalgene Company, Rochester, NY). Disinfected eggs or J2 of *H. glycines* were prepared as above. The effects of TSB-culture filtrates on egg hatch and J2 viability were tested following the same procedure as described for the bacterial cell suspensions except that both sterile water and TSB served as controls, and the 25 isolates were tested in groups of three or four. Controls were included for each test. Each isolate was tested twice.

Six suppressive bacterial isolates were further tested to see if they produced toxins when cultured in soybean root exudate. These isolates were selected for the experiment based on reduction of *H. glycines* cyst numbers by their TSB-culture filtrates. To obtain root exudates, soybean cv. Hutcheson seeds were germinated in vermiculite in a greenhouse. After 4 weeks, vermiculite was removed from the roots with tap water and the roots were rinsed three times in sterile deionized water. Soybean root exudates were collected by placing 100 of these seedlings into a sterile beaker with 1 liter of sterile deionized water. After 24 hours at room temperature (22 to 24 °C), the plants were removed and the solution containing the root exudates was sterilized by filtration through a 0.2-µm-pore cellulose nitrate membrane. For each bacterial isolate, a cell suspension was made as described and 10 ml of the suspension was mixed with 90 ml of the sterile soybean root exudates in a 150-ml flask. The mixture was incubated at 28 °C in a rotary shaker (150 rpm) for 24 hours and then centrifuged at 4 °C at 10,242*g* for 15 minutes. The supernatant was sterilized by filtration through a 0.2-µm-pore cellulose nitrate membrane and was designated modified root exudate. A 1-ml disinfected egg or J2 suspension was added to 5 ml of a modified exudate in a sterile petri dish and incubated as previously described. The treatments were replicated five times. Sterile water and unmodified sterile root exudates served as controls. After 2 (or 4) days of incubation, J2 (or eggs) were poured into soil around roots of 2-week-old seedlings of soybean cv. Hutcheson planted in heat-treated silt loam soil in 7-cm-diam. clay pots. Pots were completely randomized on a greenhouse bench maintained at 22 to 32 °C. Four weeks later, the cysts were extracted and counted. The experiment was repeated once.

Effect of organic amendments on nematode suppression by bacteria: The six bacterial isolates selected for the root exudate experiment were tested for their effect on *H. glycines* numbers in heat-treated silt loam soil amended with 0.1% (w/w) peptone (Difco Laboratories, Detroit, MI) or casein hydrolysate (Fluka Chemical Corp., Ronkonkoma, NY). Peptone and casein hydrolysate were chosen as organic amendments because they were ingredients of the media used to isolate those bacteria and would likely be used as a nutritional source for the bacteria in soil. A bacterial inoculum and a predetermined amount of peptone or casein hydrolysate (0.1% of the dry soil by weight) were added to soil of known dry weight and mixed thoroughly by hand. The final bacterial concentration in soil was 10^7 to 10^8 CFU/g dry soil. The mixed soil was put in 7-cm-diam. clay pots, and a 7-day-old seedling of soybean cv. Hutcheson was transplanted into each pot. Each treatment had five replicates. Controls were soil without bacteria or amendments and soil with only peptone or casein hydrolysate added. Pots were completely randomized on a greenhouse bench maintained at 22 to 32 °C. After 4 days, each pot was infested with 2,000 eggs of *H. glycines* by the same method as described in the greenhouse screening tests. Four weeks later, plant heights were measured, the cysts were extracted and counted, and then cysts were crushed and eggs and J2 were counted. Isolates that reduced numbers of cysts and (or) eggs and J2 of *H. glycines* were retested once.

Bacterial identification: The 20 suppressive and five enhancing bacterial isolates used in the greenhouse tests were identified based

on analysis of fatty acid methyl esters (FAMEs), which were prepared following the MIDI manual (Anonymous, 1996) and processed with the Microbial Identification System (MIS) of MIDI (Newark, DE). FAME peaks were named by the MIS software, and bacterial isolates were identified using the MIS ''main aerobic bacteria library'' and ''clinical library.''

Statistical analysis: All data were subjected to analysis of variance using SAS general linear models procedure, and differences among treatment means were separated with Fisher's least significant difference test at $P \leq 0.05$ (SAS Institute, Inc., Cary, NC).

Results

Greenhouse screening tests: Of 201 bacteria isolated from soybean roots, 138 had no effect on the numbers of cysts or eggs + J2 of *H. glycines* in the initial greenhouse test; of those that did affect nematode numbers, 36 reduced (suppressive isolates) and 27 increased (enhancing isolates) numbers of cysts and (or) eggs + J2 when compared to the control ($P \leq 0.05$). Bacterial isolates were selected for retesting from among those that reduced numbers of cysts and (or) eggs + J2 by ≥50% and from among those that increased numbers of cysts and eggs + J2 by > 100% (Table 1). Of the 20 suppressive isolates that were tested twice more, only one reduced eggs + J2 number in the second test and only four in the third test (Table 1). Of the five enhancing isolates retested, only one increased eggs + J2 number in the second test and two in the third test (Table 1). No isolate consistently in-

TABLE 1. Percentage difference in numbers of cysts and eggs + second-stage juveniles (J2) of *Heterodera glycines* in heat-treated silt loam soil infested with rhizobacteria in the greenhouse.

	Percentage difference[b]					
	Test 1		Test 2		Test 3	
Bacterial isolate[a]	Cysts	Eggs + J2	Cysts	Eggs + J2	Cysts	Eggs + J2
T1	−58*[c]	−76*	−13	−22	−13	−25
T4	−57*	−54	−7	−24	−34*	−45*
T14	−23	−55*	−29*	−29	−13	−21
T15	−85*	−92*	−27*	−30*	−13	−17
T30	−56	−53*	−15	−17	−24*	−33*
P1	−58*	−76*	−17	−23	−20*	−24
P3	−32*	−55*	−6	−7	−28*	−42*
P27	−23	−51*	−7	+1	−46*	−54*
P32	−24	−56*	−23*	−13	−1	+10
P57	−44*	−52*	−10	−3	+7	+19
P58	−67*	−70*	−13	+3	−13	+1
P59	−60*	−68*	−25*	−21	+5	+27
P62	−52*	−50*	−7	+2	+7	+14
P63	−46*	−50*	−16*	+3	+6	+27
P64	−45*	−50*	+18	+22	0	−3
P66	−44	−50*	−3	+21	−2	+24
P68	−54*	−51*	−3	+8	−21	−19
S30	−22	−60*	+2	+8	+9	+48*
S32	−17	−54*	−5	+10	−32*	−15
S38	−21	−57*	+8	+13	−31*	+14
T7	+160*	+119*	+9	+9	+23	+71*
T37	+358*	+1117*	+8	+15	−25*	−51
P11	+216*	+190*	−2	+8	−8	−6
P31	+165*	+248*	+16	+24	+15	+79*
P37	+435*	+495*	+9	+27*	−2	+11

[a] Bacteria were isolated from the rhizosphere and rhizoplane of soybean plants in Arkansas.

[b] Percentage difference = difference between means of treatment and control × 100 ÷ control mean. − = decrease, and + = increase. Each treatment (or control) was replicated five times in Test 1, 12 times in Test 2, and 10 times in Test 3.

[c] Asterisk indicates that the mean of cyst or eggs + J2 numbers is different from the control ($P \leq 0.05$).

creased or decreased nematode numbers in all three tests.

Effects of bacterial cell suspensions on egg hatch and J2 viability: When J2 were incubated in bacterial suspensions at 24 °C for 48 hours, all isolates inhibited J2 mobility ($P \leq 0.05$), except isolates P3 and P11 in Test 1 and P59 in Test 2 (Table 2). No isolate was lethal to J2 (data not shown). When J2 were applied to soybean seedlings following a 48-hour incubation in cell suspensions, the number of cysts produced was not different from the water control for any of the 25 isolates, except that isolate P3 increased and isolate P58 decreased the number of cysts in the first trial (data not shown).

When eggs were incubated in bacterial suspensions at 24 °C for 10 days, 12 of the 20 suppressive isolates inhibited egg hatch ($P \leq 0.05$), two isolates had no effect on hatch, and six had variable effects on hatch (Table 2). Of the five enhancing isolates, four decreased egg hatch and one had variable effects on hatch (Table 2). All isolates were recoverable on TSA or PAF after incubation for 10 days. When eggs were incubated in bacterial suspensions for 4 days and then applied to soybean seedlings, cyst numbers were higher with isolates T14, P58, and P11 in both trials, while cyst numbers were variable or not affected by the other isolates (Table 3).

Effects of culture filtrates on egg hatch and J2 viability: When J2 were incubated in cell-free TSB-culture filtrates for 48 hours, culture filtrates from all isolates reduced J2 mobility compared to the water control but not compared to the TSB control (data not shown). The TSB control itself reduced J2 mobility compared to the water control. None of the

TABLE 2. Effects of cell suspensions of 25 bacterial isolates on mobility of second-stage juveniles (J2) and hatch of eggs of *Heterodera glycines*.

Bacterial isolate	Percentage difference in mobile J2 number[a]		Percentage difference in egg hatch[a]	
	Test 1	Test 2	Test 1	Test 2
T1	−47*[b]	−50*	−97*	−91*
T4	−38*	−46*	−81*	−80*
T14	−26*	−42*	−34*	−29*
T15	−37*	−47*	−35*	−13
T30	−26*	−45*	−33*	−15
P1	−21*	−35*	−12	−6
P3	−13	−34*	−31*	−7
P27	−44*	−30*	−46*	−44*
P32	−31*	−32*	−57*	−52*
P57	−32*	−20*	−58*	−41*
P58	−14*	−19*	−31*	−17
P59	−19*	−11	+7	+7
P62	−30*	−25*	+30*	+4
P63	−20*	−15*	−43*	−28*
P64	−19*	−37*	−40*	−49*
P66	−19*	−17*	+6	+33*
P68	−44*	−37*	−88*	−80*
S30	−57*	−26*	−98*	−99*
S32	−65*	−54*	−85*	−89*
S38	−65*	−66*	−100*	−99*
T7	−62*	−85*	−100*	−100*
T37	−55*	−42*	−53*	−48*
P11	−7	−36*	−24*	−16
P31	−32*	−21*	−53*	−41*
P37	−38*	−33*	−58*	−28*

[a] Percentage difference = difference between means of treatment and water control × 100 ÷ water control mean. − = decrease, and + = increase. Each treatment (or control) was replicated five times.

[b] Asterisk indicates that the mean number of mobile J2 or the mean percentage egg hatch is different from the corresponding control ($P \leq 0.05$).

TABLE 3. Percentage difference in numbers of cysts of *Heterodera glycines* formed from the eggs incubated with bacterial suspensions for 48 hours and then poured into soil around soybean roots.

Bacterial isolate	Percentage difference in cyst number[a]	
	Test 1	Test 2
T1	+32*[b]	+24
T4	+11	+8
T14	+58*	+33*
T15	−12	+2
T30	+10	−2
P1	−18	+46*
P3	+34*	+25
P27	+29	0
P32	−3	+32*
P57	−16	+52*
P58	+34*	+39*
P59	+2	+37*
P62	+23	+16
P63	+25	+28
P64	+24	+21
P66	+36*	+16
P68	−15	+49*
S30	+14	+20
S32	+21	+22
S38	+13	−7
T7	+7	0
T37	+13	+20
P11	+50*	+54*
P31	−4	+43*
P37	+31	+36*

[a] Percentage difference = difference between means of treatment and water control × 100 ÷ water control mean. − = decrease, and + = increase. Each treatment (or control) was replicated five times.
[b] Asterisk indicates that the mean of cyst number is different from the water control ($P \leq 0.05$).

culture filtrates affected J2 viability (data not shown). When J2 were applied to soybean seedlings following a 48-hour incubation in TSB-culture filtrates, the number of cysts produced was lower ($P \leq 0.05$) with culture filtrates from isolates P1, P11, P59, and P64 compared to the TSB control (Fig. 1). Incubation of J2 in TSB-culture filtrates from the other isolates did not affect the number of cysts (data not shown). The TSB control had no influence on cyst numbers compared with the water control. Incubation of eggs in cell-free TSB-culture filtrates for 10 days did not affect egg hatch compared to the TSB control except filtrates from isolates P32 and P64, which had higher hatch rates than the TSB control (data not shown). The TSB control inhibited egg hatch by more than 95% in all the trials compared to the water control. When eggs were incubated in TSB-culture filtrates for 4 days and then applied to soybean seedlings, cyst numbers were reduced in culture filtrates from nine isolates (P1, P3, P37, P57, P62, T30, S30, S32, and S38) and the TSB control in both trials compared to the water control, but only culture filtrates from S30, S32, and S38 reduced the number of cysts when compared to both water and TSB controls (Fig. 2). Culture filtrates from the other bacterial isolates had no effect on the number of cysts (data not shown).

The TSB-culture filtrates of six suppressive isolates (P1, P59, P64, S30, S32, and S38) reduced cyst numbers when grown in TSB; however, when cultured in soybean root exudates for 24 hours, the resulting cell-free filtrates did not reduce cyst numbers when J2 (or eggs) were incubated in filtrates for 2 (or 4) days and then applied to soybean seedlings (data not shown).

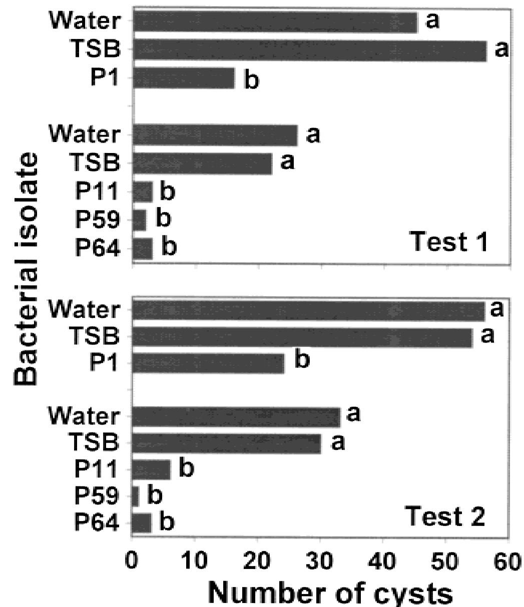

FIG. 1. Numbers of cysts formed from second-stage juveniles (J2) of *Heterodera glycines* incubated in culture filtrates of bacterial isolates derived from tryptic soy broth (TSB). The J2 were incubated for 48 hours in the culture filtrate or controls (water and TSB) before applying to soil around soybean roots. Bars are the means of five replications. Bars within a group with the same letter are not different ($P > 0.05$).

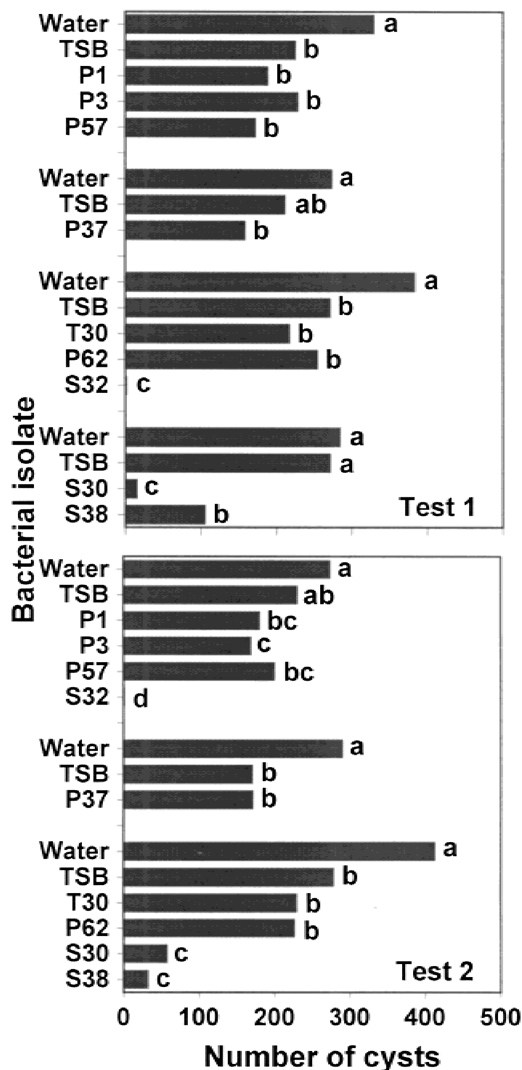

FIG. 2. Numbers of cysts formed from eggs of *Heterodera glycines* incubated in culture filtrates of bacterial isolates derived from tryptic soy broth (TSB). The eggs were incubated for 4 days in the culture filtrate or controls (water and TSB) before applying to soil around soybean roots. Bars are means of five replications. Bars within a group with the same letter are not different ($P > 0.05$).

Effect of organic amendments on nematode suppression by bacteria: Plants in soil amended with 0.1% (w/w) peptone or casein hydrolysate grew taller than those in soil without addition of the organic amendments ($P \leq 0.05$) (data not shown). In soil amended with 0.1% peptone, neither the cyst nor eggs + J2 numbers were reduced in the presence of bacterial isolates P1, S30, S32, and S38 when compared to the water or peptone-alone control (data not shown); however, the eggs + J2 numbers were reduced in the presence of isolates P59 and P64 when compared to the controls in the first trial but not in the second trial ($P \leq 0.05$) (Table 4). In soil amended with 0.1% casein hydrolysate, neither cyst nor eggs + J2 numbers were reduced when compared to the water or casein-alone control (data not shown).

Bacterial identification: Among the 20 isolates that suppressed (≥50%) nematode numbers in the initial greenhouse screening test, four were *Pseudomonas* spp., two *Bacillus* spp., three *Paenibacillus* spp., and one *Streptomyces* sp. (Table 5). The other suppressive isolates were in seven additional genera or were not identified. Among the five isolates that enhanced (>100%) nematode numbers in the initial screening test, one was *Bacillus pumilus,* one *Pseudomonas fluorescens,* and the other three were not identified (Table 5).

DISCUSSION

Potential biological control agents of soilborne plant pathogens can be screened in vitro or in soil (Merriman and Russell, 1990). In vitro screening usually selects organisms that produce toxic metabolites or that parasitize the target. Furthermore, organisms that have good antagonistic activity in vitro often have no biocontrol activity in soil (Becker et al., 1988; Neipp and Becker, 1999; Oostendorp, 1986; Racke and Sikora, 1992). Therefore, in this study, the initial screening was conducted in a heat-treated silt loam soil in the greenhouse because this screening strategy would allow for selection of multiple mechanisms of antagonism including antibiosis, competition, and parasitism. Heat-treated soil was used to reduce the risk of plant disease from pathogens in the soil. In the initial screening test, the effects of rhizobacteria on the production of cysts and eggs + J2 of *H. glycines* ranged from inhibition to stimulation. Similar phenomena have been reported with other rhizobacteria and plant parasitic nematodes (Becker et al., 1988; Oostendorp and Sikora, 1989; Racke and Sikora, 1992). However, when we re-

TABLE 4. Numbers of cysts and eggs + second-stage juveniles (J2) of *Heterodera glycines* in a heat-treated silt loam soil that had been amended with 0.1% (w/w) peptone and infested with a bacterium.

	Test 1		Test 2	
Treatment[a]	No. cysts	No. eggs + J2	No. cysts	No. eggs + J2
Blank control	277b	67,584a	145a	14,520a
Peptone control	355a	72,348a	135a	10,773a
P59	259b	49,536b	121a	8,520a
P64	226b	49,152b	133a	11,856a

Data are means of five replications. Within a column, data followed by the same letter are not different ($P > 0.05$).
[a] Blank control is a treatment without addition of peptone or a bacterial isolate; peptone control contains peptone but no bacteria.

tested some of the isolates in the same soil, the results were inconsistent. For example, isolate T15 reduced the number of eggs + J2 by 92% in the initial screening test and by 30% in the second test but had no effect on nematode numbers in the third test. Isolate T37 increased the number of eggs + J2 by more than 10 times in the initial test but had no effect in the second test and decreased the number of eggs + J2 by 51% in the third test.

A greater understanding of the ecology and modes of action of nematode antagonists may lead to more consistent biological control. Little is known of the mechanisms of nematode suppression by antagonistic rhizobacteria (Sikora and Hoffmann-Hergarten, 1993). Production of specific en-

TABLE 5. Identification of bacterial isolates by analysis of fatty acid methyl esters.

Isolate designation	Location[a]	Scientific name	Similarity index[b]
T1	Crawford Co.	*Bacillus cereus*	0.837
T4	Crawford Co.	*Paenibacillus macerans*	0.780
T14	Crawford Co.	*Pantoea citrea*	0.391
T15	Crawford Co.	*Bacillus pumilus*	0.877
T30	St. Francis Co.	*Alcaligenes piechaudii*	0.718
P1	Crawford Co.	*Pseudomonas putida*	0.495
P3	Crawford Co.	*Pseudomonas chlororaphis*	0.892
P27	Crawford Co.	*Paenibacillus lentimorbus*	0.838
P32	St. Francis Co.	Not identified	
P57	Lee Co.	*Pseudomonas putida*	0.394
P58	Lee Co.	*Pseudomonas putida*	0.411
P59	Lee Co.	*Variovorax paradoxus*	0.727
P62	Lee Co.	*Acinetobacter genospecies*	0.746
P63	Lee Co.	*Cellulomonas cartae*	0.607
P64	Lee Co.	*Hydrogenophaga pseudoflava*	0.512
P66	Crawford Co.	*Burkholderia glathei*	0.419
P68	Mississippi Co.	*Paenibacillus lentimorbus*	0.771
S30	Faulkner Co.	Not identified	
S32	Faulkner Co.	*Streptomyces violaceusniger violaceusniger*	0.484
S38	Lee Co.	Not identified	
T7	Crawford Co.	Not identified	
T37	St. Francis Co.	*Bacillus pumilus*	0.628
P11	Crawford Co.	*Pseudomonas fluorescens*	0.670
P31	St. Francis Co.	Not identified	
P37	St. Francis Co.	Not identified	

[a] County in Arkansas from which each isolate was obtained from the rhizosphere or rhizoplane of soybean plants.
[b] Similarity index is derived from the number of standard deviations that the unknown differs from the fatty acid profile of a known organism. In general, values of 0.5 or higher are considered a good match.

zymes, toxic metabolites, or modification of root exudates of the host plant may reduce egg hatch, attraction, or host recognition by nematodes (Oka et al., 1993; Sikora and Hoffmann-Hergarten, 1993). To determine whether toxins were involved in suppression of *H. glycines,* in vitro tests were conducted with bacterial suspensions and culture filtrates. Eggs and J2 were chosen for this study because these two stages are thought to be the most vulnerable to toxic metabolites from bacteria. The fact that the cell suspensions of all isolates tested, regardless of whether they suppressed or enhanced nematode numbers in soil, immobilized J2 but did not reduce their viability or infectivity suggests a general phenomenon such as low oxygen levels. Therefore, immobilization of J2 by bacterial suspensions may not be a reliable indicator of potential biocontrol agents. Bacterial suspensions of most of the suppressive and enhancing isolates inhibited egg hatch of *H. glycines.* However, cyst numbers were not reduced by any of the isolates when eggs were incubated for 4 days in bacterial suspensions and then applied to soybean seedlings, which indicates that the inhibition of egg hatch in vitro was temporary and the viability of the eggs was not affected by the bacterial suspensions.

Culture filtrates of all isolates derived from TSB reduced the mobility of J2. However, the TSB control also immobilized J2, suggesting that some chemical property of the medium contributed to the immobilization of J2. Culture filtrates of all isolates and the TSB control immobilized J2, but infectivity of the J2 was reduced only by isolates P1, P11, P59, and P64. These four isolates may have produced metabolites toxic to J2. The effect of TSB-culture filtrates on egg hatch was difficult to evaluate because the TSB control substantially reduced egg hatch. Nevertheless, isolates S30, S32, and S38 appeared to produce toxins in the culture broth because the number of cysts was reduced compared to the controls when eggs were incubated for 4 days in culture filtrates of these isolates before applying to soybean plants. Although isolates P1, P59, P64, S30, S32, and S38 produced metabolites toxic to eggs or J2 in vitro, these isolates did not consistently suppress numbers of *H. glycines* in soil. Racke and Sikora (1992) observed a similar lack of correlation between toxin production in vitro and biocontrol activity in soil. Moreover, these isolates did not seem to produce a toxin when cultured in soybean root exudate, suggesting that the toxin production is probably nutrient-dependent.

Organic matter may help establish a biocontrol agent in soil by acting as an energy source for the agent (Kerry, 1988). In the present study, little improvement in biocontrol activity of the bacterial isolates was observed with either peptone or casein hydrolysate. Perhaps the concentrations of the amendments were not sufficiently high or they were not suitable sources of nutrition for the bacteria.

For most of the isolates, the effects of their cell suspensions and culture filtrates on egg hatch or J2 viability in vitro were repeatable. However, results from the greenhouse experiments were extremely variable. No isolate consistently suppressed or enhanced nematode numbers when added to soil. Similar variable results have been reported by other researchers (Becker et al., 1988; Neipp and Becker, 1999; Oostendorp and Sikora, 1989; Racke and Sikora, 1992). The reasons for the inconsistent results are unclear. One factor that may contribute to variable results is the degree of establishment of bacteria on roots (Becker et al., 1988). Intergeneric and interspecific competition between rhizobacteria may influence bacterial colonization on the root surface (Racke and Sikora, 1992). Environmental conditions such as moisture, temperature, and plant nutritional status also may contribute to inconsistent biocontrol with rhizobacteria (Kloepper et al., 1989; Weller, 1988). In addition, bacterial traits essential for nematode antagonism may have been lost due to spontaneous mutations on artificial growth media (Duffy and Defago, 2000; Stanier et al., 1970). Variable results with biocontrol agents applied to soil have long been recognized, but little research has been done to elucidate the contributing factors.

We screened rhizobacteria for their ability to reduce numbers of *H. glycines* on soybean planted in heat-treated soil. This approach, while inclusive of different mechanisms of antagonism, was time- and space-consuming. Moreover, we were unable to identify potential biocontrol agents. The in vitro bioassays we used to determine the effect of rhizobacteria on egg hatch and J2 viability were rapid, space-efficient, and repeatable; however, these assays are not able to detect bacteria that suppressed nematode populations by modifying root exudates or by inducing systemic resistance in the host plant. Furthermore, we found no clear relationship between toxin production in vitro and antagonistic activity in soil. Although many nematode-antagonistic rhizobacteria have been identified, no standard screening system has been developed. Future research should be directed toward establishing more efficient and repeatable screening systems, and elucidating the ecology of rhizobacteria.

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