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Control of Soybean Cyst Nematode by Chitinolytic Bacteria with Chitin Substrate[1]

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Abstract: Sixty-four chitinolytic bacterial isolates from soybean fields in Arkansas were tested for suppression of soybean cyst nematode (*Heterodera glycines*) in a heat-treated silt loam soil amended with 0.6% (w/w) chitin in a greenhouse. Five isolates consistently reduced numbers of *H. glycines* compared to controls receiving neither chitin nor bacteria, or only chitin. Four of the five isolates interacted with the chitin substrate to enhance its effectiveness in reducing numbers of the nematode. The size of the clear-zone produced by some of the isolates in colloidal chitin medium, an indication of chitinolytic activity in vitro, was not related to suppression of nematode numbers in soil.

Key words: biological control, chitin, chitinase-producing bacteria, chitinolytic bacteria, *Glycine max, Heterodera glycines,* soybean, soybean cyst nematode.

Biological control tactics, including the use of chitin and chitinolytic organisms, are being evaluated as management options for plant-parasitic nematodes. Chitin is a polysaccharide, made up largely of N-acetyl-D-glucosamine, which is widely found in nature. It is an important constituent of fungi, algae, and arthropods (Muzzarelli, 1977). Chitin is also found in the middle layer of egg shells of tylenchoid nematodes such as *Meloidogyne javanica, Rotylenchulus reniformis, Tylenchulus semipenetrans,* and *Pratylenchus minyus* (Bird and McClure, 1976) and in the outer layer of egg shells of *Heterodera schachtii* and *H. glycines* (Perry and Trett, 1986).

Amendment of soil with chitin to control pathogens (*Fusarium solani* and *F. oxysporum*) was first suggested by Mitchell and Alexander (1961). The nematicidal effects of chitin on plant-parasitic nematodes was first investigated by Mankau and Das (1969, 1974), who found that chitin amendments controlled the citrus nematode *T. semipenetrans* and the root-knot nematode *M. incognita.* Later, chitin amendments were used to control *P. penetrans* and *Tylenchorhynchus dubius* (Miller et al., 1973), *M. arenaria* (Mian et al., 1982), *M. javanica* (Spiegel et al., 1987), *H. glycines* (Rodríguez-Kábana et al., 1984), and *H. avenae* (Spiegel et al., 1989).

In soil, chitin is depolymerized by chitinase resulting in the release of ammonia, which is nematicidal at certain concentrations. Addition of chitin to soil may stimulate the growth of bacteria, actinomycetes, and a limited number of fungal species with chitinolytic properties. These microorganisms may attack nematode eggs and egg masses, thus reducing populations of nematodes (Rodríguez-Kábana, 1986; Spiegel et al., 1987). However, sufficient time must elapse after chitin is added for populations of chitinolytic organisms to increase to levels adequate for effective nematode control. Consequently, nematode control is frequently better with the second crop than with the first (Rodríguez-Kábana and Morgan-Jones, 1987).

Chitinase reportedly killed *T. dubius* by producing structural changes in the nematode cuticle (Miller and Sands, 1977). Purified chitinase inhibited egg hatch of *Globodera rostochiensis* by up to 70% in vitro, and the chitinase-producing bacteria *Stenotrophomonas maltophilia* and *Chromobacterium* sp. reduced egg hatch of that nematode both in vitro and in soil (Cronin et al., 1997). *Pseudomonas chitinolytica,* with strong chitinolytic activity, reduced *M. javanica* infection and improved growth of tomato, *Lycopersicon esculentum* (Spiegel et al., 1991). The chitinolytic fungus, *Paecilomyces lilacinus,* destroyed nematode eggs and efficiently controlled *M. incognita* (Morgan-Jones et al., 1984). Furthermore, application of both *P. lilacinus*

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and chitin to sterilized soil suppressed *M. incognita* population levels more than using either alone (Mittal et al., 1995).

We hypothesized that chitinolytic bacteria would enhance the effectiveness of chitin for control of soybean cyst nematode, a serious pest of soybean in Arkansas and other regions of the United States. Although a few chitinolytic microorganisms have been found to suppress nematode numbers, it was unknown whether they would colonize soybean roots or suppress soybean cyst nematode. The rhizosphere is the first line of defense for roots against attack by soilborne pathogens (Weller, 1988). Rhizosphere colonization by introduced microorganisms is essential for biocontrol of root pathogens (Suslow, 1982). Presumably, chitinolytic bacteria isolated from the rhizosphere and rhizoplane of soybean would be good colonizers. Therefore, the objectives of this study were to: (i) isolate chitinolytic bacteria from the rhizosphere of soybean, (ii) examine their effects on soybean cyst nematode with a chitin substrate, and (iii) identify bacterial isolates that were able to enhance the effectiveness of chitin amendment for control of this nematode.

Materials and Methods

Isolation and storage of chitinolytic bacteria: Soil samples were arbitrarily collected from soybean fields in Faulkner, Greene, Johnson, Lafayette, Lonoke, Pope, and St. Francis counties in Arkansas. Each sample (600 ml) was mixed with 1% (w/w) ground chitin (Sigma Chemical Co., St. Louis, MO) and put in 10-cm-diam. clay pots. Two 7-day-old soybean, *Glycine max* (L.) Mer. cv. Hutcheson, seedlings were transplanted into each pot and maintained in a greenhouse at 22 to 32 °C. Two weeks later, the roots of the soybean plants were gently removed from the pots to retain the soil adhering to the roots (rhizosphere soil). Bacteria from the rhizoplane and rhizosphere of the plants were isolated on colloidal chitin media at pH 7.0 (Williams and Wellington, 1982) by the method of Wollum (1982), except that the diluent was buffered water (Greenberg et al., 1992). From each soil sample, bacterial colonies that produced an obvious clearzone in the medium and that differed in color and/or morphology were purified by streaking the culture on TSA medium to get a single colony. Pure cultures of bacterial isolates were stored in cryogenic vials (Nalgene Company, Rochester, NY) containing sterile tryptic soy broth (Sigma Chemical Co., St. Louis, MO) with 20% sterile glycerol at −76 °C.

Nematode inoculum: Heterodera glycines race 3 was cultured on susceptible soybean cv. Lee 74 in a greenhouse at 22 to 32 °C by pouring eggs plus second-stage juveniles (J2) into the soil surrounding the roots. After 5 weeks, cysts were extracted by sieving and sugar flotation (Southey, 1986). Eggs and J2 for experiments were obtained by crushing cysts with a ground-glass tissue grinder and collecting the contents on a 25-µm-pore sieve nested under a 75-µm-pore sieve.

Greenhouse screening: Bacteria were grown on tryptic soy agar (TSA, pH 7.3 ± 0.2) overnight at 30 °C. Bacterial cells were swabbed from the agar surface with sterile cotton-tipped applicators and placed in sterile deionized water. Isolates not easily swabbed from the agar surface were cultured in tryptic soy broth in 500-ml flasks at 30 °C on a rotary shaker (150 rpm) for 48 hours. The culture was then centrifuged at 4 °C and 3,000*g* for 15 minutes, the supernatant was decanted, and the bacterial pellet was resuspended in sterile deionized water.

Bacterial cell concentrations were determined at ×1280 magnification with a hemocytometer and adjusted to 10^9 CFU per ml. Seeds of soybean cv. Hutcheson were germinated in vermiculite. Roots of 7-day-old seedlings were washed free of vermiculite in running tap water, rinsed in sterile deionized water three times, and immersed in a bacterial suspension or sterile water (control) for 15 to 20 minutes. The seedlings were then planted singly in a 7-cm-diam. clay pot containing 280 ml soil amended with 0.6% (dry weight) ground chitin. The soil was a fine silt loam from the Arkansas River Valley (42.1% sand, 54.9% silt, 3% clay; pH

7.6; and 0.5% organic matter) that was heat-treated at 105 °C for 3 hours and used throughout this research. An additional 10-ml aliquot of the bacterial suspension was added to soil around roots. Plants receiving neither chitin nor bacteria and plants receiving chitin but not bacteria served as controls. Treatments were replicated five times and arranged in a completely randomized design in a greenhouse maintained at 22 to 32 °C. After 4 days, two small holes were made in the soil adjacent to the base of the seedling with sterile pipet tips, and 1,000 eggs + J2 of *H. glycines,* which had been washed in sterile deionized water three times, were added to each hole. The holes were filled with a small volume of soil without chitin. After 4 weeks, cysts were extracted from all plants, crushed as described earlier, and the eggs + J2 were counted 4 weeks later.

A two-step screen was used to identify bacterial isolates that were most effective in reducing numbers of *H. glycines*. Isolates that reduced the number of eggs + J2 by at least 80% compared to the control receiving neither chitin nor bacteria in the first step were tested again. Each isolate was replicated five times per step. Bacterial isolates that reduced the number of eggs + J2 compared to either of the controls in the second step were included in the experiment. The experimental methods were the same as the two-step screen except that each treatment was replicated 10 times and plant heights were recorded at the time of data collection. The experiment was performed three times.

Comparison of chitinolytic activity of selected bacterial isolates: Bacterial isolates that suppressed numbers of *H. glycines* at least twice compared to either of the controls in the greenhouse experiment were tested to determine if nematode suppression was related to their chitinolytic activity. Fifteen-ml aliquots of sterilized TSA or colloidal chitin medium were introduced into 100 × 15-mm sterile plastic petri dishes. A loop of a fresh bacterial suspension was put on the TSA medium and evenly spread on the surface using a sterile cotton-tipped applicator. After incubation at 30 °C for 24 hours, three 0.9-cm-diam. agar plugs were removed with a sterile cork borer. Each plug was placed, colony-side down, in the center of a dish containing colloidal chitin medium and incubated at 30 °C. The diameter of the clear-zone in each petri dish was measured after 7 days. The experiment was repeated once.

Different levels of chitin substrate: Bacterial isolates that consistently suppressed numbers of *H. glycines* in the greenhouse experiment, compared to either of the controls, were evaluated for their effects on *H. glycines* numbers and plant growth in soil amended with 0, 0.01%, 0.3%, 0.4%, and 0.5% (w/w) chitin. A suspension of each bacterial isolate was prepared as described earlier and mixed thoroughly by hand with 2,700 g of dry soil containing different percentages of chitin. The final bacterial concentration in the soil was 10^7 to 10^8 CFU per g dry soil. Treatments with soil containing neither chitin nor bacteria, and treatments with only chitin added, served as controls. The mixed soil was then put in 7-cm-diam. clay pots, and a 7-day-old Hutcheson soybean seedling was transplanted into each pot. Each treatment was replicated 10 times. Pots were completely randomized on a bench in a greenhouse maintained at 22 to 32 °C. Four days later, each pot was infested with 2,000 eggs + J2 of *H. glycines* by the same method described in the greenhouse screening experiment. After 4 weeks, plant height was measured, cysts of *H. glycines* were extracted and crushed, and the number of eggs + J2 were counted. The trials for different chitin levels were conducted at different times due to limitations of labor and greenhouse space. For each chitin level, the trial was done twice and included the two controls.

Bacterial identification: Isolates that suppressed numbers of *H. glycines* at least twice, compared to either of the controls, in the greenhouse experiment were identified based on analysis of fatty acid methyl esters (FAME), 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.'' Isolates that were not identified by FAME analysis were identified to genus level based on their cultural type and morphology.

Statistical analysis: All the data were subjected to analysis of variance using the SAS general linear models procedure, and differences among treatment means were separated by Fisher's least significant difference test at $P \leq 0.05$ (SAS Institute, Inc., Cary, NC). Regression analysis was used to determine rate of reduction in nematode numbers for each bacterial isolate and the control receiving chitin only (chitin control). The rate of reduction is equal to the slope of the nematode numbers plotted against chitin amendment level. Prior to analysis, the numbers were transformed to log10 and then, within each trial, divided by the mean number of nematodes in the control receiving neither chitin nor bacteria to correct for differences in nematode reproduction between trials. A *t*-test was used to determine whether the slopes for the bacterial isolates differed from the chitin control. An isolate with a different slope from the chitin control would indicate an interaction between the isolate and chitin substrate.

Results

Greenhouse screening: In the first step of the screen, numbers of *H. glycines* in controls receiving chitin alone were extremely variable. Therefore, treatments were compared to the more consistent control that received neither chitin nor bacteria. Of the 64 bacterial isolates tested, 15 isolates reduced the number of eggs + J2 of *H. glycines* by at least 80% compared to the control receiving neither chitin nor bacteria. In the second step of the screen, seven isolates, designated as C6, C10, C11, C15, C19, C31, and C54, reduced the numbers of eggs + J2 of *H. glycines* compared to controls receiving neither chitin nor bacteria or receiving chitin only ($P \leq 0.05$). When these seven isolates were tested three more times, only five isolates (C6, C10, C11, C31, and C54) consistently suppressed reproduction of the nematode compared to either of the controls, while isolates C15 and C19 had variable effects (Fig. 1). Plant height was neither consistently increased or decreased by either chitin alone or chitin plus any of the bacterial isolates compared to the control without chitin or bacteria.

Comparison of chitinolytic activity of selected bacterial isolates: On colloidal chitin medium, isolates C15 and C19 exhibited the strongest and chitinolytic activity and isolates C6 and C54 the weakest (Table 1).

Different levels of chitin substrate: For all the bacterial isolates and the chitin control, there was a decrease in the number of nematodes with increasing chitin levels ($P <$

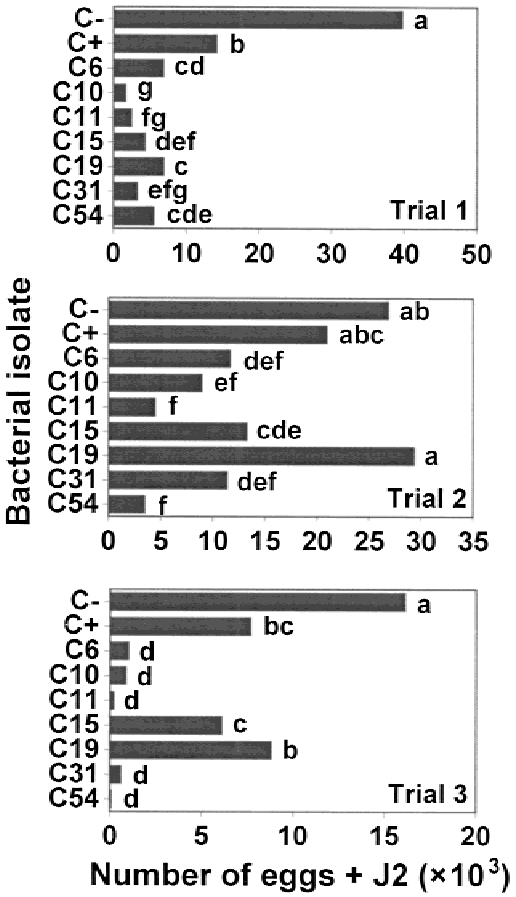

Fig. 1. Numbers of eggs+second-stage juveniles (J2) of *Heterodera glycines* in a heat-treated silt loam soil amended with 0.6% (w/w) chitin and infested with chitinolytic bacteria. C– = control that had neither chitin nor bacteria added; C+ = control that had chitin but not bacteria added. Bars are means of 10 replications, and bars with the same letter are not different ($P > 0.05$) according to Fisher's least significant difference test.

TABLE 1. Diameters of clear-zones produced by chitinolytic bacteria in colloidal chitin medium.

Isolate	Diameter of clear-zone (cm)	
	Trial 1	Trial 2
C6	2.30c	2.15c
C10	2.55b	2.62b
C11	2.60b	2.52b
C15	4.37a	4.48a
C19	4.31a	4.57a
C31	2.65b	2.57b
C54	2.30c	2.13c

Data are means of three replications. Data followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference test.

0.0001; Table 2). The rate of reduction (i.e., slope) in numbers of *H. glycines* was greater for isolates C10, C11, C31, and C54 than for isolate C6 or the chitin control ($P \leq 0.05$). None of the isolates affected plant height when soil had 0 or 0.01% chitin (data not shown). However, at the 0.3% chitin level, plant height was greater in soil treated with isolate C10 or C31 than in the control receiving neither chitin nor bacteria, but not different from the chitin control (data not shown). At the 0.4% and 0.5% chitin levels, the effect of bacterial isolates on plant height varied from positive to negative (Table 3). Isolates C6 and C10 consistently reduced plant height in soil amended with 0.5% chitin.

Bacterial identification: Five species from four genera (*Comamonas, Flavobacterium, Methylobacterium, Streptomyces*) were identified from among the seven selected isolates (Table 4). The other two isolates were unidentified *Streptomyces* species.

TABLE 2. Rate of reduction of *Heterodera glycines* by chitinolytic bacteria in soil amended with different levels of chitin.[a]

Isolate	Rate of reduction Slopes ± SE[b]	P-value	R^2
Chitin control	−0.2223 ± 0.0509	0.0001	0.20
C6	−0.6082 ± 0.1013	0.0001	0.30
C10	−1.6183 ± 0.1501*	0.0001	0.57
C11	−1.1380 ± 0.1321*	0.0001	0.46
C31	−1.1087 ± 0.1488*	0.0001	0.39
C54	−0.7587 ± 0.0998*	0.0001	0.40

[a] The soil was heat-treated and amended with 0, 0.01, 0.3, 0.4, or 0.5% chitin. Soil receiving neither chitin nor bacteria and soil receiving chitin only (chitin control) served as controls. Regression analysis was used to determine rate of reduction (i.e., slope) in nematode numbers for each bacterial isolate and the chitin control.

[b] Asterisk indicates that the slope for an isolate is different from that for the chitin control according to *t*-test ($P \leq 0.05$).

TABLE 3. Plant height in a heat-treated silt loam soil amended with 0.4% or 0.5% (w/w) chitin as affected by chitinolytic bacterial isolates in a greenhouse.

Isolate[a]	Plant height (cm)			
	0.4% chitin		0.5% chitin	
	Trial 1	Trial 2	Trial 1	Trial 2
C–	15.9a[b]	20.7bc	15.9a	23.6ab
C+	16.1a	23.3ab	15.2a	26.2a
C6	13.3c	18.6c	11.3c	18.6d
C10	11.3c	22.3ab	11.0c	19.1cd
C11	16.0a	22.9ab	14.0ab	22.0bcd
C31	14.9ab	22.2ab	12.9bc	24.5ab
C54	13.8b	23.8a	15.6a	22.5bc

[a] C– = control that had neither chitin nor bacteria added to the soil; C+ = control that had chitin but not bacteria added to the soil.

[b] Data are means of 10 replications. Within a column, data followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference test.

DISCUSSION

Random screening of microorganisms for biocontrol of plant-parasitic nematodes' without regard to characteristics related to nematode antagonism' has a low probability of success because of the large number of organisms that must be tested (Becker et al., 1988; Tian, 1999). Based on the information that chitinase and chitinolytic microorganisms play a significant role in the control of plant-parasitic nematodes following chitin amendments (Rodríguez-Kábana, 1986; Spiegel et al., 1987), we tested a group of Chitinolytic bacteria for their effect on *H. glycines* in chitin-amended soil. Using this approach, we found five chitinolytic bacteria that consistently suppressed numbers of *H. glycines'* and four of these enhanced the effectiveness of the chitin substrate. Our results support the suggestion of Mittal et al. (1995) that other microorganisms, in addition to *P. lilacinus,* may be able to increase the effectiveness of chitin amendments for nematode control. However, this enhancement of chitin was not observed for all chiti-

TABLE 4. Identities of bacterial isolates based on analysis of fatty acid methyl esters.

Bacterial isolate	Location[a]	Identification	Similarity index[b]
C6	Pope Co.[1]	*Streptomyces cyaneus chartreusis*	0.466
C10	Pope Co.[1]	*Methylobacterium zatmanii*	0.857
C11	Pope Co.[1]	*Streptomyces* sp.	
C15	Pope Co.[2]	*Flavobacterium johnsoniae*	0.777
C19	Pope Co.[3]	*Flavobacterium johnsoniae*	0.343
C31	Lonoke Co.	*Comamonas acidovorans*	0.843
C54	St. Francis Co.	*Streptomyces* sp.	

[a] County in Arkansas in which isolates were obtained from soybean field soil. County names with the same number indicate that the bacterial isolates were from the same field. Otherwise, they were from different fields.

[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.

nolytic bacteria tested. Even among the five consistently effective isolates, isolate C6 did not interact with the chitin substrate. Perhaps the reduction of *H. glycines* by this isolate and the chitin substrate was simply additive.

In the absence of bacterial isolates, amendment of the soil with chitin alone at rates of 0.4 to 0.6% (w/w) had variable effects on reproduction of soybean cyst nematode. This variation may be related to soil pH, or to the amount and kinds of microorganisms remaining in the soil or recolonizing the soil after heat treatment. Although the same soil was used throughout the study, changes in the microbial community may have resulted in different rates of chitin degradation and, thus, different ammonia concentrations and soil pH. Rodríguez-Kábana et al. (1984) observed similar inconsistencies in nematode suppression with soil amendments of less than 1% chitin. However, soil amendments of greater than 1% chitin are usually phytotoxic.

There was no apparent relationship between the chitinolytic activity of the bacterial isolates, as indicated by the size of the clearzone produced in colloidal chitin medium, and suppression of *H. glycines* in chitin-amended soil. Similarly, Cronin et al. (1997) reported that inhibition of egg hatch in vitro by chitinase-producing bacteria was not correlated with the amount of chitinase produced on chitin medium. Perhaps the bacteria produce different types of chitinase or other factors in addition to chitinase production are involved in antagonism of nematodes (Mercer et al., 1992).

The bacterial isolates in combination with chitin substrate had variable effects on plant height. Although uniform seedlings were selected during transplanting, many other factors such as light quality, watering, soil microbial community, soil pH, and survival of applied bacteria could cause the variation. The occasional adverse effects of some isolates on plant growth may have been due to rapid degradation of chitin and release of a high concentration of ammonia into the soil. Ammonia can go through the nitrification process to become nitrites and nitrates—all of which are toxic to plants at high concentration (Rodríguez-Kábana et al., 1984; Spiegel et al., 1987). This phytotoxicity can be reduced or avoided by including an easily metabolizable source of carbon together with the chitin to broaden the C:N ratio (Culbreath et al., 1985; Spiegel et al., 1986).

We found five chitinolytic bacterial isolates that consistently suppressed numbers of *H. glycines* when applied with a chitin substrate. Moreover, four of these isolates enhanced the effectiveness of chitin amendment for control of the nematode. However, to better use these isolates, more research is needed to determine their exact mode of action against nematodes, their survival in soil, and efficient formulation and application methods.

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