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Evaluation of Biocontrol Activity of Rhizobacteria from *Beta vulgaris* against *Heterodera schachtii*[1]

P. W. Neipp and J. O. Becker[2]

Abstract: One hundred fifty rhizobacteria isolated from roots of Swiss chard grown in a soil suppressive to the sugar beet cyst nematode, *Heterodera schachtii,* were tested for their influence on the nematode's ability to hatch and infect roots. Two screening systems were used that focused on the ability of bacteria to inhibit either nematode hatching or root infection. Most of the bacterial strains reduced hatching in vitro compared to the control, while with 5% of the strains there were 0% hatch. Seven percent of all strains significantly reduced second-stage juvenile (J2) infection of mustard roots raised in soil-less growth pouches. Eighteen strains from four genera (*Bacillus, Pseudomonas, Variovorax,* and *Arthrobacter*) were selected for greenhouse trials. In the greenhouse trials four bacterial strains, including two strains of *Bacillus megaterium,* reduced nematode infection of sugar beet when eggs were used as inoculum. Seven bacterial strains reduced nematode infection of sugar beet in one of two trials when plants were inoculated with J2. Most of the effective strains were *Bacillus* spp., primarily *B. megaterium.* Colonization of sugar beets roots by two *B. megaterium* strains was found to be stable over 30 days in the greenhouse.

Key words: Bacillus megaterium, Beta vulgaris, biological control, *Heterodera schachtii,* nematode, rhizobacteria, sugar beet, sugar beet cyst nematode.

The range of organisms identified as potential biocontrol agents of plant-parasitic nematodes is extensive and includes fungi, bacteria, soil invertebrates, and predatory nematodes (Stirling, 1991). Although an encounter between a nematode and its antagonist may occur anywhere in the soil, certain developmental stages of most plant-parasitic nematodes commonly occur in the root zone of plants. For example, potential targets for disruption are nematode eggs and first-stage juveniles, hatching, juvenile movement through the soil, attraction to roots, recognition of host or non-host tissue and feeding sites, attraction to opposite gender, and penetration of root tissues. These critical stages are exposed to countless interactions among roots of host or non-host plants, soil biota, and the physical and chemical environment of the rhizosphere. Of the soil biota, bacteria are the most abundant microorganisms in the root zone. The presence of specific naturally-occurring or introduced rhizobacteria can significantly modify the rhizosphere environment environment and affect directly or indirectly the nematode or the host-parasite interrelationship. Consequently, rhizobacteria have been evaluated for their effects on a variety of plant-parasitic nematodes, including *Meloidogyne incognita* (Becker et al., 1988, 1989; Kloepper et al., 1992; Zavaleta-Mejia, 1985), *M. hapla* (Honglin et al., 1995), *M. javanica* (Spiegel et al., 1991), *Criconemella xenoplax* (Kluepfel et al., 1993), *Heterodera glycines* (Kloepper et al., 1992), *H. schachtii* (Oostendorp and Sikora, 1989, 1990), *Globodera pallida* (Racke and Sikora, 1992), and *G. rostochiensis* (Cronin et al., 1997). Many rhizobacteria, including strains of *Serratia* (Zavaleta-Mejia, 1985), *Pseudomonas* (Becker et al., 1989, Oostendorp and Sikora, 1989, 1990, Kluepfel et al., 1993), and *Bacillus* (Becker et al., 1988), have been effective antagonists to plant-parasitic nematodes.

Although several specific mechanisms by which rhizobacteria inhibit fungal or bacterial plant pathogens have been demonstrated (Weller, 1988), few have been reported against nematodes. Some bacterial metabolites, such as avermectins (Stretton et al., 1987), valinomycin (Mishra et al., 1987), and 2,4-diacetylphloroglucinol (Cronin et al., 1997), and volatile metabolites such as various organic acids, hydrogen sulfide, and ammonia, have adverse effects on nema-

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[2] Graduate Assistant and Associate Nematologist/Associate Cooperative Extension Specialist, Department of Nematology, University of California, Riverside, CA 92521.

E-mail: ole.becker@ucr.edu

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todes (Stirling, 1991), but their occurrence and significance in situ are disputed. The objective of this research was to detect and identify rhizobacteria that show a detrimental effect on eggs and second-stage juveniles (J2) of *H. schachtii* Schmidt in vitro and reduce early root infection in greenhouse trials.

Materials and Methods

Isolation of rhizobacteria from Swiss chard grown in a soil suppressive to H. schachtii: One hundred fifty rhizobacteria strains were isolated from roots of ten 3 to 6-week-old Swiss-chard (*Beta vulgaris* L. 'Paros') grown in field 9E at the University of California Riverside Agricultural Experiment Station. Isolations were done at three separate times during a 30-day period in fall 1995. The soil in this field is suppressive to *H. schachtii,* and the biological nature of the suppressiveness has been demonstrated recently (Westphal and Becker, 1996). Roots were shaken vigorously to remove most of the adhering soil. Roots from individual plants were placed in 9 ml of sterile deionized water (SDW) and ground in a sterile mortar and pestle. One milliliter of the homogenate was diluted 10-fold serially to 10^{-5} of the original concentration. After discarding the 10^{-1} dilution, dilutions 10^{-2} and 10^{-3} were heated in 70 °C water bath for 15 minutes and plated on potato dextrose agar (Difco Laboratories, Detroit, MI) to isolate spore-forming bacteria. Unheated dilutions 10^{-4} and 10^{-5} were plated on King's medium B (King et al., 1954) to isolate a variety of bacteria. After 24 to 48-hours' growth on plates, individual colonies were streaked again for purity, grown for 24 to 48 hours in 10% tryptic soy broth (TSB; Difco Laboratories, Detroit, MI), and stored in sterile 50% glycerol at –80 °C.

Hatching assay: Bacterial test strains were grown in TSB for 48 hours and then centrifuged at 13,600 g for 5 minutes. The supernatant, suspected of containing potentially anti-nematode metabolites, was aseptically removed and placed into 96-well microtiter plates (150 µl/well). Each well also received 150 µl of a hatching stimulant, 3 mM zinc chloride (Clarke and Shepherd, 1966). The control consisted of wells containing 150 µl SDW plus 150 µl of the hatching stimulant. Cysts of *H. schachtii* were surface-sterilized with 1.3% NaOCL for 7 minutes (Heungens et al., 1996), and one cyst was placed in each well with 3 wells/treatment. Occasionally, bacterial growth occurred in some test wells; these wells were not included in the evaluation. Each well was adjacent to a well containing 0.05 M sulfuric acid to trap toxic ammonia potentially remaining in the supernatant. After 3 days, hatching was evaluated by counting the total number of J2 outside of each cyst. Trapped ammonia was detected with Nessler's reagent. Bacterial strains that completely inhibited hatching were tested two more times.

Growth pouch assay: The growth pouch screen employed was a modification of a previously developed technique (Becker et al., 1988). Mustard seeds, *Sinapis alba* 'Florida Broadleaf,' were surface-sterilized in a 20% commercial bleach solution (1.05% NaOCl) for 5 minutes and rinsed three times in SDW. The 150 bacterial test strains were grown individually in TSB for 48 hours on a TC-7 rollerdrum (New Brunswick Scientific, Edison, NJ) at 25 °C and centrifuged at 13,600 g for 5 minutes. The pellets were washed in SDW and resuspended in 2% methyl cellulose (~10^9 cfu/ml). Each seed was coated with a bacterial suspension, sown in a growth pouch amended with 1 ml full-strength Hoagland's solution, and thoroughly wetted with SDW. Pouches were randomized in a growth chamber at 28 °C/23 °C and a 16-hour/8-hour day/night cycle. There were three replicates per treatment. Controls consisted of plants not treated with bacteria or nematodes and plants in pouches infested only with nematodes. After 3 days, each pouch received either 300 or 500 J2 of *H. schachtii* (depending on the trial) in 1 ml of SDW placed on the back side of the pouches opposite the root and along the root length. Pouches were incubated horizontally in the dark for 24 hours to facilitate nematode root infection. Seven days after inoculation, roots were stained with

acid fuchsin and observed for nematode infection (Byrd et al., 1983).

Greenhouse tests of selected bacterial strains: Eighteen bacterial strains effective in the previous two screens were evaluated further in greenhouse trials. Eight of these strains that inhibited hatch (0% hatch) were tested for effects when eggs were used as inoculum. One of those strains (42) plus 10 others that significantly reduced J2 infection of mustard roots in growth pouches were tested with J2 as inoculum. Bacterial were grown for 72 hours in mannitol glutamate broth (MG) (Keane et al., 1970), centrifuged at 2,000 g for 15 minutes, and resuspended in SDW. Sugar beet ('HH77') was seeded into 10 × 10-cm fiber pots (four seeds/pot) filled with 600 cm^3 of steam-pasteurized fine sand. Pasteurized soil was chosen to provide some measure of reproducibility for these experiments (Kempf and Becker, 1992) even though it became recolonized by greenhouse-resident fungi and bacteria. Each pot was drenched with 25 ml of bacterial suspension (~10^7 cfu/ml), while the control received only 25 ml of SDW. After seedling emergence (approximately 4 to 5 days), plants were thinned to one per pot and each pot was infested either with 3,000 nematode eggs or with J2 (trial 1 1,000; trial 2 700). Roots were stained as described above for nematode detection 8 days after infestation with J2 and 14 days after inoculation with eggs. The design of both experiments was a randomized complete block with seven replications per treatment. Each experiment was repeated once. The greenhouse was maintained at 24 to 27 °C and received sunlight but no artificial lighting. Each plant was fertilized with 0.5 g slow-release fertilizer (17-6-10, Osmocote, Sierra Chemical, Milpitas, CA) and watered twice each day.

Root colonization of sugar beet: Two strains of *B. megaterium,* 42 and 60, previously used in the greenhouse studies, were evaluated for root colonization capability on sugar beet in pasteurized, but recolonized sand. Spontaneous rifampicin-resistant mutants were isolated from bacterial cultures grown in 25 ml MG broth containing 50 µg/ml rifampicin. Mutants were selected from each culture and purified by streaking on PDA amended with 50 µg/ml rifampicin. Growth of mutants was compared to that of the wild type on non-antibiotic media. Each mutant was then subjected to 15 consecutive transfers, first to non-antibiotic media and then to antibiotic media, to determine the stability of the rifampicin-resistance marker.

Bacterial inoculations were similar to those used for the other greenhouse tests. Sugar beet seeds were sown into styrofoam cups (355 ml) with 4 seeds/cup. Each cup then received 20 ml bacterial drench over the seeds (10^7 cfu/ml). The control treatment consisted of a sterile water drench but no bacterial application. Plants were arranged in the greenhouse in a completely randomized design with 6 replicates. The greenhouse was maintained at approximately 24 °C, and plants received sunlight but no artificial lighting. Seedlings were thinned to one per cup after emergence. Each plant was fertilized with 0.5 g slow-release fertilizer (17-6-10), and plants were watered twice each day. At 5-day intervals beginning 5 days after seedling emergence, one group of plants was removed from the experiment and the roots were gently washed with tap water. Roots were weighed after being blotted dry and were ground in a mortar and pestle in 3 to 12 ml SDW, depending on the root mass. Serial dilutions were then spotted onto fresh PDA plates amended with 50 µg/ml rifampicin. Plates were incubated at 30 °C in the dark for 24 hours before colonies were counted. The experiment lasted 30 days and was repeated once.

Identification of bacterial strains: The 18 strains used in the greenhouse studies were identified with fatty acid methyl ester analysis (FAME). Bacterial growth and fatty acid preparations were done according to the MIDI Manual (Anonymous, 1996). None of the other strains was identified.

Statistical analysis: For in vitro hatching results, statistical analysis was performed with an unpaired *t*-test. Data from growth pouch and greenhouse experiments were analyzed with ANOVA and Fisher's Protected Least Significant Difference (LSD) test at $P \leq$

0.05). Data from greenhouse trials with *H. schachtii* egg inoculum were transformed with log $(x \pm 1)$ and combined before statistical analysis with ANOVA.

Results

Influence of rhizobacteria on nematode hatching: One hundred thirty-nine of the 150 strains significantly reduced hatching of J2 from cysts relative to hatching in the control. Only one strain stimulated hatching compared with the control. Eight strains inhibited hatching (0% hatch): strain 12 (*Variovorax paradoxus*); strain 38 and 89 (both *Bacillus pumilus*); strain 42, 60, and 96 (all *Bacillus megaterium*); strain 155 (*Pseudomonas pseudoalcaligenes*); and strain 157 (*Arthrobacter oxydans*). With 7% of the strains, hatching was comparable to the hatching stimulant alone. Eighty-seven percent of the strains inhibited hatching less than 50%. Ammonia was produced by 57 strains, but there was no correlation between inhibition of hatching and ammonia production.

Influence of rhizobacteria on root infection in growth pouches: Of the 150 bacterial strains tested, 11 strains significantly reduced nematode root infection in growth pouches (Fig. 1). The percent reduction in infection ranged from 33% to 72%. Two strains increased penetration, while the remaining strains did not significantly affect root infection. Five strains significantly increased root length as compared with the check but did not reduce nematode root infection (data not shown).

Effectiveness of selected bacteria in the greenhouse: Four strains (42, 96, 155, and 157) significantly reduced nematode numbers per gram of root when sugar beet seedlings were inoculated with eggs (Fig. 2). Strain 42 reduced nematode numbers per gram of root by 50% and 74%, and strain 96 reduced nematodes per gram of root by 71% and 61%, respectively, in trials 1 and 2.

Results were mixed when J2 were used as inoculum. No strain significantly reduced nematode infection in the first trial. In the second trial, strains 6, 11, 34, 40, 42, 108, and 121 reduced nematode numbers in roots 38% to 59% compared to controls (Table 1).

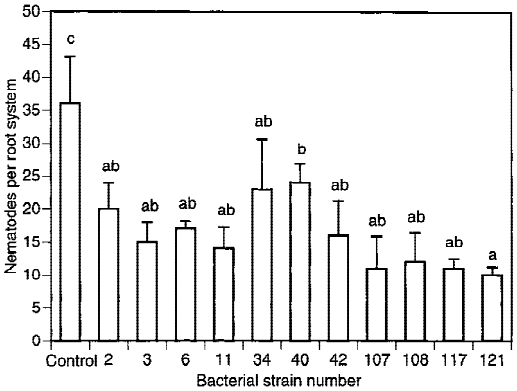

Fig. 1. Reduction of *Heterodera schachtii* second-stage juvenile (J2) infection of mustard roots in growth pouches after treatment of seeds with rhizobacteria. Results are shown from the 11 strains that reduced nematode root infection. Nematodes were counted in stained roots 7 days after inoculation with J2. Lines above bars indicate SE values for seven replications. Bars with a common letter are not significantly different ($P \geq 0.05$) according to Fisher's Protected Least Significant Difference Test.

Comparison of bioassays: All of the eighteen strains used in the greenhouse trials inhibited hatching at least 30% in the microtiter plate assay (Table 2). Although strains 12 and 60 inhibited hatching, they had no effect on J2 infection of mustard roots in growth pouches. Three strains (3, 89, and 107) had no effect on the nematode in

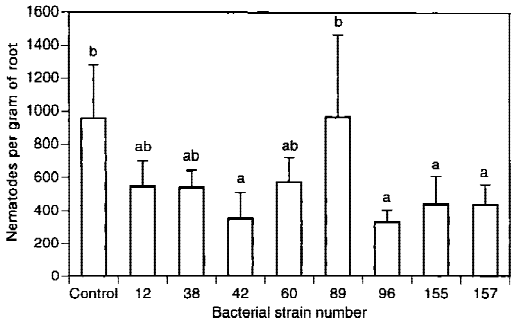

Fig. 2. Numbers of *Heterodera schachtii* found in roots 14 days after inoculating each sugar beet plant with 3,000 eggs. Data from two trials were transformed with log $(x+1)$ and combined before statistical analysis with ANOVA. Back-transformed data are presented. Lines above bars indicate SE values for seven replications. Bars with a common letter are not significantly different at ($P \geq 0.05$) according to Fisher's Protected Least Significant Difference Test.

TABLE 1. Effect of rhizobacteria on invasion of sugar beet roots by second-stage juveniles (J2) of *Heterodera schachtii* in greenhouse trials.

Treatment[a]	Nematodes per gram of root	
	Trial 1	Trial 2
Control	2,037 abc	434 c
2	1,604 abc	358 bc
3	2,479 bc	365 bc
6	1,742 abc	252 ab
11	2,078 abc	215 ab
34	1,053 a	247 ab
40	1,224 ab	268 ab
42	1,216 ab	237 ab
107	2,749 c	307 abc
108	1,852 abc	181 a
117	1,096 ab	288 abc
121	1,354 ab	179 a

Data are means of seven replications. Within a column, data followed by the same letter are not significantly different ($P \geq 0.05$) according to Fisher's Protected Least Significant Difference test.

[a] Numbers indicate a particular bacterial strain. Bacteria were applied as a drench (25 ml of ~10^7 cfu/ml bacterial suspension in SDW) over sugar beet seeds after planting into 10 × 10-cm pots filled with pasteurized fine sand. Pots were infested with 1,000 *Heterodera schachtii* J2 (trial 1) or 700 J2 (trial 2).

greenhouse trials although they negatively influenced the nematode in the in vitro assays. Nine strains (2, 3, 6, 11, 42, 89, 107, 108, and 117) had a progressively diminishing impact on the nematode when the assays incorporated a host or soil was used. However, some strains (12, 34, 38, 40, 60, 96, 155, and 157) were more effective in the greenhouse than in pouches.

TABLE 2. Effect of 18 bacterial isolates in 3 different bioassays on hatching of *Heterodera schachtii* eggs.

Bacterial strain	Percent inhibition of *H. schachtii*		
	Hatching assay	Pouch test	Greenhouse tests
2	97	45	21
3	99	58	0
6	77	53	19
11	77	61	7
12	100	0	52
34	93	36	47
38	100	25	54
40	93	33	40
42	100	56	41
60	100	0	47
89	100	33	0
96	100	53	70
107	93	69	0
108	90	67	18
117	77	69	44
121	30	72	38
155	100	36	54
157	100	19	62

Bacteria populations: Populations ranged from 4.1 to 5.6 log cfu/g root for strain 42 and from 4.6 to 5.8 log cfu/g root for strain 60 (Fig. 3). Strains 42 and 60 increased in population throughout the experiment, reaching their highest populations on day 30 for strain 42 and on day 25 for strain 60. The population levels of strains 42 and 60 remained very similar over 30 days. However, populations of strains 42 and 60 were significantly higher (at least 1 log unit higher) at day 25 than at day 10. Low background levels of native bacteria resistant to rifampicin were detected in the check.

Bacteria identification: Seven different species of bacteria from four genera (*Bacillus, Pseudomonas, Variovorax,* and *Arthrobacter*) were identified among the 18 strains selected for greenhouse trials (Table 3). Twelve strains were *Bacillus* spp., primarily *B. megaterium.*

DISCUSSION

Nematode-suppressive soils are potentially a rich source of microorganisms active against plant-parasitic nematodes (Stirling, 1991). The bacterial strains tested in this study were isolated from roots of Swiss chard

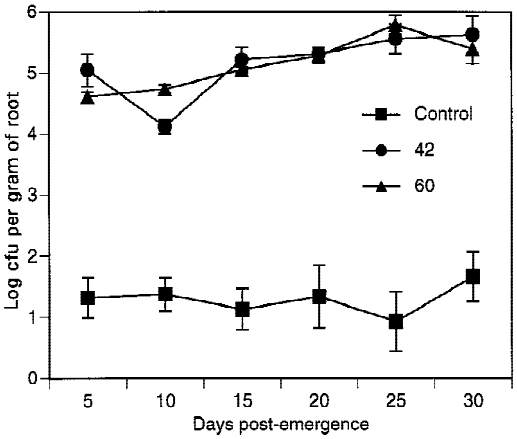

FIG. 3. Population levels of two bacterial strains (42, 60) on roots of sugar beet over 30 days in the greenhouse. Roots were sampled every 5 days beginning 5 days after seedling emergence. Cfu = colony-forming units.

TABLE 3. Identification by fatty acid methyl ester analysis of rhizobacteria used in greenhouse studies.

Bacterial strain no.	Identification	Similarity index[a]
2	*Bacillus brevis*	0.627
3	*Variovorax paradoxus*	0.703
6	*Arthrobacter oxydans*	0.510
11	*A. ramosus*	0.711
12	*V. paradoxus*	0.355
34	*B. megaterium*	0.709
38	*B. pumilus*	0.775
40	*B. megaterium*	0.596
42	*B. megaterium*	0.729
60	*B. megaterium*	0.588
89	*B. pumilus*	0.728
96	*B. megaterium*	0.854
107	*B. megaterium*	0.586
108	*B. megaterium*	0.629
117	*B. pumilus*	0.597
121	*B. megaterium*	0.494
155	*Pseudomonas pseudoalcaligenes*	0.871
157	*A. oxydans*	0.637

[a] Percent similarity of these strains compared to fatty acid profiles of known organisms. Values of >0.5 are considered a good match.

grown in a sugar beet cyst nematode-suppressive field, and the majority of these strains reduced hatching of *H. schachtii* juveniles in vitro. Virtually all well-described rhizobacteria with excellent biocontrol activity have shown in vitro production of antimicrobial metabolites, and in situ production of these metabolites is of major importance in the biological control of pathogens (Schroth and Becker, 1990). For example, in a similar study, a strain of root-colonizing *Pseudomonas aureofaciens* isolated from a soil suppressive to *Criconemella xenoplax* inhibited *C. xenoplax* egg hatch in vitro but also reduced ring nematode populations in greenhouse trials (Westcott and Kluepfel, 1993). These results indicate that selection of an antagonist based on production of metabolites in artificial media may be a useful initial screening procedure to reduce the number of candidate strains for testing in greenhouse experiments. However, the fact that the supernatant of many bacterial strains had an inhibitory effect on egg hatch in the current study suggests a rather general, unspecific mechanism in the in vitro test. Effects caused by microbial production of broad-spectrum biocides such as ammonia were minimized by gas trapping. Still, the results seem to confirm the notion that selection of biocontrol agents on the basis of in vitro antibiosis is impractical, since only a small percentage of antibiotic-producing strains also have biocontrol activity (Jacobsen and Backman, 1993). It is unclear what selection criteria should be applied to successfully isolate nematode-antagonistic bacteria. Kloepper et al. (1992) suggested that nematode-antagonistic bacteria may be more abundant in the rhizosphere of nematode-antagonistic plants. Yet a higher percentage of strains from susceptibile soybean plants were effective against *M. incognita* than were strains from plants such as castor bean, sword bean, and Abruzzi rye that are antagonistic to the nematode. The opposite situation was found with *H. glycines,* suggesting that the same bacteria strains may differ in their biocontrol activity against different nematodes.

Additionally, the task of finding bacterial strains with antagonistic activity against nematodes is difficult because of the scarcity of reference strains. Reference strains are especially valuable in adjusting a screening process and optimizing methodology toward a particular pest. For example, most described strains with activity against nematodes are either very specific to a certain species or have not been placed in depositories to allow general access. Researchers investigating biological control of fungal diseases by rhizobacteria frequently address this problem by utilizing well-described strains such as *Pseudomonas fluorescens* 2-79 (Weller and Cook, 1983) or *Bacillus subtilis* A13 (Broadbent et al., 1977).

In this study, 7 of 11 strains that reduced J2 root infection in pouches also reduced nematode root infection in a greenhouse trial with J2 used as inoculum. The growth pouch screen was chosen to rapidly select rhizobacteria that could negatively influence root infection. One advantage of this technique is that bacteria utilize root-derived nutrients as the sole energy source (Becker et al., 1988). Thus, any bacterium that has a negative effect on the nematode is

likely to thrive in this simplified environment. The test is also useful in detecting strains that have a negative effect on plant growth.

The initial screening in the present study focused exclusively on the selection of biologically active bacteria. Selected candidates were further tested in greenhouse trials. Most of the strains identified as potential antagonists in this study and used in the greenhouse trials were *Bacillus* spp., primarily *B. megaterium*. *Bacillus* spp. commonly reside on sugar beet roots where they may comprise 57% to 100% of the total number of bacteria recovered (Stanghellini and Rasmussen, 1989). Furthermore, *B. megaterium* can extensively colonize the rhizosphere as shown for strains 42 and 60 in this study. Likewise, strain B153-2-2, a biocontrol agent used against *Rhizoctonia solani* on soybean, was found to increase in population over a 4-week period (Liu and Sinclair, 1993). Bacilli are considered to be good biocontrol candidates for soil-borne pests such as nematodes since, under unfavorable conditions, they form long-lived spores capable of germinating into active cells under more conducive conditions. The ability to survive as spores is also a desirable trait in view of commercial shelf-life requirements for biocontrol products (Becker and Schwinn, 1993). Additionally, various strains of *B. megaterium* produce antibiotic compounds (Vary, 1992), although no compounds from *B. megaterium* have been reported with activity against nematodes. This study demonstrates that *B. megaterium* has potential as a promising biocontrol candidate against nematodes.

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