Effects of Rhizobacteria on Root-Knot Nematodes and Gall Formation

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This research was supported in part by a grant from the Ciba-Geigy Corporation.
The authors thank L. A. Gudmundson and T. A. Hoban for excellent technical assistance.
Accepted for publication 20 May 1988 (submitted for electronic processing).

ABSTRACT

Becker, J. O., Zavaleta-Mejia, E., Colbert, S. F., Schroth, M. N., Weinhold, A. R., Hancock, J. G., and Van Gundy, S. D. 1988. Effects of rhizobacteria on root-knot nematodes and gall formation. Phytopathology 78:1466-1469.

Three hundred and fifty-four randomly selected bacteria from plant rhizospheres, when tested for activity against *Meloidogyne incognita*, caused a wide range of effects from a reduction to an increase of root galling on tomato and cucumber in greenhouse tests. Results were highly variable, even with strains that previously had given significant differences. A bioassay, based on selecting bacterial strains that produced nematicidal compounds in vitro, proved to be a better and more rapid means of identifying promising nematode antagonists. About 1% of more than 5,000 bacteria isolated from rhizospheres of different plants produced detectable compounds that affected the vitality of second-stage juveniles of *M. incognita* in an in vitro test. Twenty percent of these subsequently reduced the number of galls on cucumber in a soil-free pouch system when applied as a seed treatment. Selected strains were applied as a drench to nonsterile soil infested with *M. incognita*. White clover plants growing in bacteria-treated soil had fewer galls and larger root systems. Both plant top and root weights were significantly greater compared with the nontreated control.

Additional keywords: bacterization, biological control.

Biological control of nematodes has long been considered an alternative to managing nematodes with pesticides (24). This field has developed slowly because of the past successes of chemicals and the relatively low effort given to searching for alternatives. Interest in alternatives has increased greatly in the last few years because of costs and environmental and health concerns associated with the use of chemicals. Some of these chemicals have affected animals and humans and have been detected in ground waters.

Past research on biological control of plant parasitic nematodes mainly has focused on the isolation and use of parasites and predators of nematodes (10,13,21) that occur in soils throughout the world. Such agents unquestionably play an important role in the balance of nematode populations. Nematophagous fungi often show promise in retarding nematode damage in greenhouse tests in steamed soil, but they have not been very effective in field soils. Fungistasis, slow growth, and the requirement for nematode hosts before the fungi sporulate and produce traps make nematophagous fungi relatively unattractive for practical field application (10).

In the past, researchers have claimed that bacteria are deleterious to nematodes, especially after organic material has been incorporated into the soil (20), or under anaerobic soil conditions (8,19). *Pasteuria penetrans* (= *Bacillus penetrans*) (12) has been identified as a parasite of nematodes with interesting possibilities. However, a major obstacle to its use is that it is an obligate parasite and has not been cultured. In other studies, compounds toxic to saprophytic and plant parasitic nematodes have been found in the culture broth of certain actinomycetes and other bacteria (7,9,17,25).

The discovery of a group of new biocides, avermectins, produced by *Streptomyces avermitilis* (4) provides hope that other soil organisms may produce compounds with similar potential (24). Accordingly, we examined rhizobacteria for their potential to produce compounds that inhibit plant parsitic nematodes. The approach was similar to that used to identify bacteria that caused plant growth promotion or were biologically active against fungal pathogens (22). We describe screening procedures to identify rhizobacteria that produce water-soluble sustances that affect *Meloidogyne incognita* (Kofoid and White) Chitwood and evaluate the efficacy in reducing galling of plant roots by the nematode. Brief reports of this study have been published (1,27).

MATERIALS AND METHODS

Isolation, culture, and identification of bacteria. Bacteria were isolated from the rhizospheres of several plant species collected from various locations in California. Roots were washed in 0.1 M phosphate buffer, and appropriate dilutions were plated on King's medium B (KMB, pH 7), 10% tryptic soy agar (TSA, pH 7.2) (Difco Laboratories, Detroit, MI), and potato-dextrose agar (PDA, pH 5.6) (Difco Laboratories). Randomly selected candidate strains were purified and stored in 40% glycerol at −80 C. Gram-negative strains of interest were identified with API 20E test strips (Analytap Products, Ayerst Labs, Inc., Plainview, NY) and additional tests (11).

Initial greenhouse tests. Bacteria were grown on nutrient agar for 2 days at 29 C and suspended in deionized sterile water (about 10^9 colony-forming units [cfu]/ml). The root system of 10-day-old tomato (*Lycopersicon esculentum* Mill. 'Tropic') or cucumber seedlings (*Cucumis sativus* L. 'Spacemaster') were immersed in a bacterial suspension or sterile water (control) and planted in 200-ml drinking cups with a sandy soil from a Coachella Valley, CA, grape vineyard, which was heavily infested with root-knot nematodes. Soil was mixed with pasteurized loamy sand to establish a population density of approximately 500 second-stage juveniles of *M. incognita* per cup. The experimental design was a completely randomized block with 10 replicates per treatment. Slow-release fertilizer, 4 g/cup, 17-6-10, Osmocote (Sierra Chemical Co., Milpitas, CA), was added to the cups after 1 wk. After 4 wk in the greenhouse at 24± 3 C, plants were removed and the roots were gently washed free of soil. Shoot dry weight and galling of the root systems were recorded (root-knot rating on a scale of 0 to 10, with 0 = no galls and 10 = all roots severely galled) (3).

In vitro plate screening. Bacterial strains to be tested were cultured in test tubes with nutrient yeast extract broth (NYEB) and aerated by keeping them in a Rollerdrum TC-7 (New Brunswick Scientific Co., Edison, NJ) at 1 rpm for 2 days at 22 C. A loop of bacterial suspension was streaked cross-like on 10% TSA in 60× 15 mm petri dishes. Dishes were incubated at 27 C for 4–6 days, depending on the growth rate of the test organism. Three agar plugs were cut close to the center of the cross with a 5-mm-diameter sterile corkborer. Care was taken not to contact the bacterial colonies to avoid contaminating growth on the agar plugs, which were each transferred to a separate 96-well, sterile microtiter plate

having 6.4-mm-diameter wells (Cell Wells, Corning Glass Works, Corning, NY). Forty microliters of buffered 1.5% water agar (Bacto agar, Difco Laboratories, with 10 mM HEPES *N*-[2-hydroxyethyl] piperazine-*N'*-2-ethanesulfonic acid, pH 7.2, Sigma, St. Louis, MO) containing rifampicin (100 μg/ml, Sigma) was added to each well. Plates were covered with a lid, sealed with Parafilm, and incubated overnight. Nematode inoculum was obtained from 2- to 3-mo-old tomato plants, which had been inoculated with race 1 juveniles of *M. incognita* 6 wk after seeding. Nematode eggs were collected by a modified extraction technique (15). Galled roots containing egg masses were washed free of soil with tap water and cut into pieces 2 cm long. These were triturated for two 30-sec intervals at maximum speed with a two-speed blender (Waring, New Hartford, CT) in a 0.26% sodium hypochlorite solution (commercial bleach). The solution was poured through a series of screens to separate the organic matter from the nematode eggs. Eggs were collected on a 30-μm-pore sieve (Tyler, Mentor, OH) and carefully washed with tap water. The egg suspension then was poured onto a modified Baermann's apparatus (23) and incubated at 26 C. Hatched, second-stage juveniles were collected daily. Only 1-day-old nematodes were used for the experiments. They were collected by centrifugation at 120 *g* for 1 min (Dynac II Centrifuge, Clay Adams, Parsippany, NJ) and disinfested with a solution of chlorhexidine at 5 g/L (Sigma) and 100 μg of rifampicin/ml for 30 min. Nematodes were added to the microtiter plate after washing three times with sterile water. Each well received about 20 juveniles in 50 μl of sterile water. Plates were covered with a lid, sealed with Parafilm, and incubated at room temperature (approximately 22 C). The mobility of the nematodes was examined under low-power magnification at daily intervals. Each test was repeated at least once.

Pouch test. The second-stage screening was done with a modified soil-free system used for assaying nematicidal activity of chemicals (18). Cucumber seeds were disinfested in 1.05% sodium hypochlorite for 2 min and washed several times with sterile water. Bacterial strains were grown in 5 ml of NYEB for 24 hr at 23 C in a Rollerdrum. One milliliter of the bacterial suspension was delivered into a 1.5-ml microcentrifuge tube and centrifuged at 8,750 *g* (Microfuge B, Beckman Instruments, Palo Alto, CA). The bacterial pellet was resuspended in 1 ml of methyl cellulose solution (10 g/L, 4,000 centipoises, Sigma) and thoroughly mixed with 20 cucumber seeds in a sterile petri dish, resulting in a concentration of about 10^7–10^8 bacterial cells per seed. Control seeds were treated with sterile methyl cellulose solution. Seeds were immediately dried in a laminar flow hood and then seeded into seed-pack growth pouches (Northrup King Co., Minneapolis, MN), which were divided and sealed in the middle with a pouch sealer (SAM II, Dazey Products Co., Industrial Airports, KS). One seed was placed into each pouch compartment and each treatment consisted of 10 pouches. The compartments were moistened with 4 ml of plant nutrient solution (1 ml of Oxygen Plus, 4-4-4 [Plant Research Laboratories, Irvine, CA] per 380 ml of water). Pouches were arranged in a completely randomized design and placed in an upright position in a growth chamber at 26 C and 14,700 lx illumination with a 12-hr day-night cycle. After the radical had grown to approximately 6–7 cm, the seedlings were inoculated with second-stage juveniles of *M. incognita* obtained from greenhouse-grown tomato plants as described above. About 300 juveniles were suspended in 1 ml of water and evenly distributed over the backside of the paper support in the pouches. Pouches were incubated in the dark for 24 hr in a horizontal position at room temperature to avoid passive migration of the juveniles to the bottom of the pouch. They then were randomly placed in a plastic tray, which supported the pouches in a vertical position, and returned to the growth chamber. The number of root-knot galls on each root system was counted after 6–8 days.

Greenhouse tests. Eggs of *M. incognita*, obtained from 2- to 3-mo-old greenhouse-grown tomato plants as described above, were carefully mixed into sandy loam soil (1,000 eggs/75 g dry weight of soil), which had been sieved through a 3-mm-pore screen. The soil then was added to 15-cm-long × 2.5-cm-diameter plastic cones (Ray Leach, Canby, OR), which were supported in a hanging position in racks. White clover seeds (*Trifolium repens* L. 'Dutch') were disinfested in 0.53% sodium hypochlorite for 3 min followed by several rinses with sterile water. Three seeds were planted in each cone and irrigated until water drained from the bottom. Bacterial strains were grown for 2 days in potato-dextrose broth (PDB) (Difco Laboratories) in test tubes in a Rollerdrum. Ten cones per treatment each were drenched with 2 ml of a bacterial suspension containing 10^9 cfu/ml. Control cones received 2 ml of sterile PDB. Cones were placed in the racks in a randomized complete block design and incubated in the greenhouse at 26 ± 3 C. After 5 days, plants were thinned to one per cone. After 1 wk, slow-release fertilizer (Osmocote, 17-6-10) was added to the cones at 1 g/cone. The clover plants were carefully washed out of the cones onto screens after 4 wk. The amount of root-knot infection was estimated with the gall-rating system (3). In a second trial, fresh weights of tops and roots also were taken after blotting the plants between sheets of filter paper. The experiment was repeated once.

RESULTS

Initial greenhouse tests. The effects of a random assortment of rhizosphere bacteria on plant growth and root-knot infections ranged from plant stunting to increased and decreased gall formation. Many strains caused a detectable decrease in the gall index and an increase in the top dry weight. However, results were highly variable, and only 17 bacterial strains on tomato and 19 on cucumber suppressed galling significantly ($P = 0.05$). Of those, only two also increased dry weight of tomato tops, and none had a significant effect on top dry weight of cucumber. Subsequent tests with the two most effective strains yielded inconclusive, variable results.

In vitro plate screening and pouch tests. More than 5,000 rhizobacteria strains were isolated and tested in the microtiter plate test for their ability to produce compounds that affected vitality of juveniles of *M. incognita*. Although nematodes in the control wells survived at least 10 days, only those bacteria that caused a change in nematode behavior during the first 5 days of incubation were selected for further testing. About 1% of the bacterial strains produced substances that caused a partial to complete inhibition of movement of the juveniles.

Of the selected strains that showed activity in vitro, about 20% of them significantly reduced the number of galls in cucumber seedlings. Because error variances of two trials were homogeneous, an overall analysis of variance was performed on pooled data. Strains JOB 204, JOB 209, and JOB 23 consistently reduced galling compared with the nontreated control (Table 1). None of these strains significantly affected growth of the plants when nematodes were absent.

Greenhouse experiments. Root systems of white clover grown in nematode-infested soil were generally very short and heavily galled. Main roots often were inhibited early in their growth by massive galling at the tips. Root systems of plants grown in soil

TABLE 1. Effect of treating seed with bacterial strains on number of galls caused by *Meloidogyne incognita* on cucumber grown in seed pouches

Treatment[a]	Number of galls[b]		
	Experiment 1	Experiment 2	Mean[c]
Control	146	177	162 A
JOB 23	111	159	134 B
JOB 62	141	189	165 A
JOB 73	143	173	158 A
JOB 103	147	172	160 A
JOB 204	118	148	133 B
JOB 209	121	158	139 B

[a] Seeds were mixed with bacterial suspension in 1% methyl cellulose and dried before seeded (approximately 10^7–10^8 colony-forming units/seed). Control treated with methyl cellulose only.

[b] Means of 20 plants per treatment.

[c] Values followed by the same letter do not differ significantly at $P = 0.05$, according to Duncan's multiple range test.

treated with JOB 23 (Fig. 1), JOB 204, or JOB 209 generally were more extensive than those from the control. Although still severely galled, the gall index was significantly reduced by 21–36% (Table 2). Consequently, clover plants from those treatments were significantly larger than those from nontreated controls or treatments with ineffective bacteria (Table 2).

Strain identification. The three most effective rhizobacteria strains were identified as *Pseudomonas fluorescens* Migula biovar I (JOB 209) and biovar IV (JOB 204), and *Bacillus* sp. (JOB 23) (11).

DISCUSSION

The greatest problem in screening microorganisms for biocontrol of nematodes or fungi is not having the necessary information on phenotypic characters that most likely are associated with desired ecological and physiological behavior patterns. Thus, most researchers generally base their initial isolation procedures on some characteristics thought to be of importance, such as antibiotic or siderophore production (6). Our first approach to find bacteria for nematode control was to select rhizosphere bacteria at random, using no predetermined bias in the isolation procedures. This was time consuming and deemed an unsuccessful approach because of the great variability of results. Although some strains caused a significant decrease in galls and an increase in plant weight, erratic results upon repeated testings were discouraging and suggested the need for another method. We have no ready explanation for the variability but believe that it involves the establishment of the bacteria on roots. A major factor that affects galling by the nematodes is whether the bacteria are injurious to the roots. A number of the bacteria appeared to stunt roots and, thus, may have affected available sites for nematode infections.

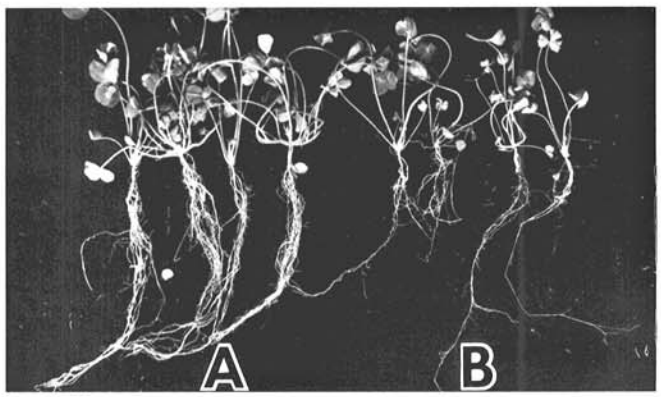

Fig. 1. Effect of bacterial drench (**A,** *Bacillus* sp. JOB 23) on white clover plants compared with the nontreated control **(B)** grown in soil infested with *Meloidogyne incognita*.

TABLE 2. Effects of soil drenching with various bacterial strains on weight of white clover plants and root galling in soil infested with *Meloidogyne incognita*

Treatment[a]	Experiment 1	Experiment 2		
	Galling index[b]	Top weight[c]	Root weight[d]	Galling index
Control 1 (no nematodes)	···	0.80 A[e]	0.43 A	···
JOB 23	5.6 BC	0.53 B	0.31 B	4.9 C
JOB 62	7.9 A	0.27 D	0.17 E	7.1 A
JOB 73	6.9 AB	0.29 D	0.25 CD	6.6 AB
JOB 103	6.4 AB	0.26 D	0.26 CD	7.6 A
JOB 204	5.0 C	0.39 C	0.30 B	5.2 C
JOB 209	6.2 B	0.41 C	0.29 BC	5.6 BC
Control 2 (with nematodes)	7.8 A	0.28 D	0.22 D	7.4 A

[a] Bacterial strains were applied as a drench of 2 ml of 2-day-old culture in potato-dextrose broth (PDB), approximately 1×10^9 colony-forming units/ml; control received 2 ml of sterile PDB. There were 20 plants per treatment.
[b] Mean of root-knot index (3).
[c,d] Mean fresh weight in grams per plant.
[e] Values within columns by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

Screening bacteria on the basis of in vitro nematicidal activity proved to be a better method. About 1% of the screened bacteria produced compounds that were inhibitory to second-stage juveniles, and 20% of them produced significant results when tested in vivo. The in vitro method enabled numerous bacterial strains to be processed with minimum requirements of equipment and space. Procedures differ somewhat from methods that others have used. Use of rich broth cultures to detect activity was rejected because some bacteria do not produce antibiotics in broth and the richness may inhibit production of the desired compound. We have observed that many bacterial strains produce detectable amounts of antibiotics in agar media, but not in broth cultures with the same nutrient composition. Agar plugs were transferred from the antibiotic-test plates to microtiter plates to avoid such volatiles as HCN and ammonia, which are readily produced (2,5,26) and cause confusing results. This has the disadvantage of diluting potentially interesting nematicidal compounds but also ensures that activity when found must be relatively potent. Plant parasitic rather than microbivorous nematodes were used as test organisms because of possible differences in sensitivity and response to toxic compounds (14,16). Because the test is still relatively crude, no effort was made to categorize the different nematode responses that were noted, such as a paralyzed reaction versus death or the quickness of the behavioral responses. The microtiter screening technique also might be useful for testing other nematode species and stages of development.

The pouch system was very useful in further screening bacteria because growth and activity of the test strains are supported only by the quantity and quality of substrates from the seed and plant root. It also could reveal an indirect effect where the bacteria affect the physiology of the root. However, selection of host plant is critical. Tomato, for example, was a diffident host in that the tap root grew very rapidly with few lateral branches for the nematode to attack. Cucumber was an ideal test plant because of the root growth pattern and its growing rate.

The same type of problem occurred with the greenhouse testing of candidate bacteria. This is considered the important test before field testing is commenced. Here, many plants, such as tomato and cucumber, grew so rapidly in the cones that maintaining a healthy root environment was not possible. Consequently, wilting or too frequent watering were found to interfere with nematode infection or the activity of the bacteria. White clover proved to be a good host for this screen.

From these studies and ongoing field investigations, it appears that the use of microbial antagonists of nematodes in plant rhizospheres could develop into a valuable crop management tool to reduce the impact of plant parasitic nematodes on plant growth. As with other microbial antagonists, successful use will depend upon having first a thorough understanding of their ecological behavior and requirements, followed by studies of their physiology and genetics.

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