Tylenchulus semipenetrans Alters the Microbial Community in the Citrus Rhizosphere

F. E. El-Borai, L. W. Duncan, J. H. Graham, and E. Dickstein

Abstract: Infection of citrus seedlings by Tylenchulus semipenetrans was shown to reduce subsequent infection of roots by Phytophthora nicotianae and to increase plant growth compared to plants infected by only the fungus. Hypothetical mechanisms by which the nematode suppresses fungal development include nutrient competition, direct antibiosis, or alteration of the microbial community in the rhizosphere to favor microorganisms antagonistic to P. nicotianae. A test of the last hypothesis was conducted via surveys of five sites in each of three citrus orchards infested with both organisms. A total of 180 2-cm-long fibrous root segments, half with a female T. semipenetrans egg mass on the root surface and half without, were obtained from each orchard site. The samples were macerated in water, and fungi and bacteria in the suspensions were isolated, quantified, and identified. No differences were detected in the numbers of microorganism species isolated from nematode-infected and uninfected root segments. However, nematode-infected root segments had significantly more propagules of bacteria at all orchard sites. Bacillus megaterium and Burkholderia cepacia were the dominant bacterial species recovered. Bacteria belonging to the genera Arthrobacter and Stenotrophomonas were encountered less frequently. The fungus community was dominated by Fusarium solani, but Trichoderma, Verticillium, Phytophthora, and Penicillium spp. also were recovered. All isolated bacteria equally inhibited the growth of P. nicotianae in vitro. Experiments using selected bacteria, T. semipenetrans, and P. nicotianae, alone or in combination, were conducted in both the laboratory and greenhouse. Root and stem fresh weights of P. nicotianae-infected plants treated with T. semipenetrans, B. cepacia, or B. megaterium were greater than for plants treated only with the fungus. Phytophthora nicotianae protein in roots of fungus-infected plants was reduced by nematodes (P ≤ 0.001), either alone or in combination with either bacterium. However, treatment with bacteria did not affect P. nicotianae development in roots. The results suggest different mechanisms by which T. semipenetrans, B. cepacia, and B. megaterium may mitigate virulence of P. nicotianae.

Key words: Bacillus megaterium, Burkholderia cepacia, interaction, nematode, Phytophthora nicotianae, rhizosphere microorganisms, Tylenchulus semipenetrans.

The interactions between plant-parasitic nematodes and other plant pathogens are commonly perceived to be indirect, the result of modifications in the host plant such as localized or systemic responses to wounding (Carter, 1981; Hillocks, 1986; Moorman et al., 1980; Westerlund et al., 1974) or other physiological alterations (Bowman and Bloom, 1966; Van Gundy et al., 1977). Root exudates reflecting the biochemical and physiological changes induced by nematode infection alter microbial community dynamics in the rhizosphere and on roots (Van Gundy et al., 1977; Wang and Bergeson, 1974; Weinhold et al., 1972). Root exudates, which can attract the motile stage of fungal pathogens (Zentmyer, 1961), represent a source of nutrients for soil microflora, stimulate the germination of dormant spores, and may be the first stage in synergistic interactions between nematodes and fungi (Taylor, 1990). For example, root leachates induced by root-knot nematodes can enhance the colonization of the rhizosphere by pathogenic fungi (Golden and Van Gundy, 1972, 1975; Kett, 1956; Khan and Muller, 1982) and reduce the numbers of actinomycetes, antagonistic to other fungi in the rhizosphere (Bergeson, 1972). However, substances that emanate directly from nematodes also have been shown to affect associated microorganisms. The gelatinous matrices surrounding eggs of the root-knot nematodes Meloidogyne javanica and M. fallax provide protection from microbial attack due either to antibiotic compounds of nematode origin or from associated bacteria (Orion and Kritzman, 1991; Kok et al., 2001).

The most common association between pathogens of the citrus fibrous root system is that between the citrus nematode T. semipenetrans Cobb and the root-rot fungus P. nicotianae Breda de Ham (synonym = P. parasitica Dastur [Hall, 1993]). Both organisms are nearly ubiquitous in citrus-growing regions. Each feeds on the cortex of fibrous roots, and both have been shown to reduce the fibrous root density (Duncan et al., 1993; Graham and Menge, 1999). Previous experiments in the field and greenhouse (Graham and Duncan, 1997) followed by a series of laboratory and greenhouse studies (El-Borai et al., 2002b) showed that the citrus nematode T. semipenetrans reduced root infection by P. nicotianae and increased growth of citrus seedlings compared with seedlings infected by P. nicotianae alone. These results imply an antagonistic interaction between T. semipenetrans and P. nicotianae.

Hypothetical mechanisms for antagonism by nematodes to fungi include direct antibiosis, competition for resources in roots, or indirect mediation through increased colonization of nematode feeding sites by microorganisms antagonistic to P. nicotianae. Following the last hypothesis, we sought to determine whether infection by T. semipenetrans changes the composition of rhizosphere-inhabiting microorganisms, and to identify microorganisms that are associated consistently with the nematode. We also conducted in vitro and

Received for publication 22 January 2002.

1 This research was supported by the Florida Agricultural Experiment Station and a grant in part from the Florida Citrus Production Research Advisory Council, and approved for publication as Journal Series No. R-8854. This is a portion of a PhD dissertation by the first author.

2 Graduate Research Assistant, Nematology, University of Florida, Institute of Food and Agricultural Sciences, Citrus Research and Education Center, Lake Alfred, FL 33850.

3 Professors, University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, FL 33850.

4 Senior Biological Scientist, Plant Pathology, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611. E-mail: fahiem@lal.ufl.edu

This paper was edited by Deborah Neher.
whole plant experiments with candidate bacterial species to determine their capacity to inhibit root infection by *P. nicotianae* in the presence or absence of the citrus nematode.

**Materials and Methods**

**Field survey:** A survey was conducted in three citrus orchards to investigate whether root infection by *T. semipenetrans* is associated with changes in communities of bacteria and fungi in the rhizosphere. Roots naturally infected by *T. semipenetrans* were collected from citrus orchards near Ona, Bartow, and Lake Alfred in central Florida. Lake Alfred and Ona were surveyed in July 1998 and March 1999; Bartow in March 1999. The groves were separated by distances ranging from 24 to 72 km. Five random trees were sampled in each orchard, and five fibrous root samples (0 to 30-cm depth) collected from each tree were composited. Six groups, each with 30 root segments (2.0 to 2.5-mm length), were prepared from each sample, placed in 5 ml distilled water, and macerated for approximately 8 seconds in a Tissuemizer (Tekmar Co., Cincinnati, OH). Half of the groups contained root segments infected by female *T. semipenetrans* with an egg mass on the root surface, and the remaining groups contained uninfected segments as identified at 10× magnification under a stereomicroscope. The resulting suspensions were diluted (10⁻¹, 10⁻², and 10⁻³), and each dilution was streaked onto five petri dishes (0.1 ml per dish) of PARP-H (corn meal agar with antibiotics), selective for *Phytophthora* (Graham, 1990; Mitchell and Kannwischer-Mitchell, 1992), and five dishes of potato dextrose agar (PDA; Difco Laboratories, Detroit, MI). After 72 hours, bacterial and fungal colonies were counted and individual colonies isolated.

Bacteria were cultured on 1.5% nutrient agar (Sigma Chemical Company, St. Louis, MO). The isolates were identified by fatty acid analysis using Fatty Acid Methyl-Esters (FAMEs) (Sasser, 1990) and the Aerobic Bacterial Library of MIDI (Microbial Identification, Newark, DE) (Anonymous, 1996). Cultures were maintained at −80 °C. Predominant fungal colonies were transferred to new nutrient agar plates within glass cylinder cells for separation from contaminant organisms by allowing the fungus to grow downward and laterally. The plates were incubated at room temperature (25 °C) for 5 days, after which the procedure was repeated. Fungi were identified to genus or species by microscopic and stereoscopic analysis, using taxonomic keys. In the first survey (July 1998) at Lake Alfred and Ona, the colony forming units (CFUs) were quantified and isolated for preliminary identification. In the second survey (March 1999), the isolated organisms were quantified and identified to species, at all three sites.

The total number of bacterial CFUs for the two surveys at Ona and Lake Alfred were analyzed by three-way analysis of variance, testing for effects of survey date, grove, type of root segment, and all possible interactions (Minitab Inc., State College, PA). In the second survey of all three sites, data for the predominant organisms were analyzed by two-way analysis of variance (ANOVA), testing for effects of site, root type, and the interaction between them. All data were transformed (log, n+1) prior to ANOVA, but untransformed means are reported.

**In vitro inhibition of *P. nicotianae* by isolated bacteria:** Bioassays were conducted to determine whether bacteria associated with *T. semipenetrans*-infected roots affect growth of *P. nicotianae*. Bacterial isolates were prepared by streaking onto nutrient agar and allowed to grow for 48 hours at room temperature. Using a bacterial loop, a single cell colony from newly grown colonies was transferred to nutrient agar in 100 × 15-mm petri dishes (Fisher Scientific, Pittsburgh, PA) by streaking the cells in a circle (3 cm wide) circumscribing the center of the dish. A 4-mm mycelial plug taken from an actively growing colony of *P. nicotianae* (isolate P-117) grown in PARP-H media was placed in the center of the dish. Ten replicate plates for each bacterium were incubated at room temperature for 3 days, after which the *P. nicotianae* colony radius was measured from eight different directions and means computed. An analysis of variance was performed and, when appropriate, followed by Dunnett’s test to compare the control treatment (*P. nicotianae* alone) with the five bacteria tested (Dunnett, 1955).

**Bacteria and the interaction between *T. semipenetrans* and *P. nicotianae**: Two experiments (greenhouse and laboratory) were conducted to determine whether rhizosphere bacteria in the presence or absence of *T. semipenetrans* affect the virulence of *P. nicotianae* on citrus. In both experiments, isolates of *Burkholderia cepacia* and *Bacillus megaterium* were used with *P. nicotianae* and *T. semipenetrans* to establish the following incomplete factorial treatments: bacteria alone; fungus alone; nematode alone; bacteria + fungus; nematode + fungus; bacteria + fungus + nematode; and untreated control. Both experiments were run with 10, single plant replicates per treatment, in a completely randomized design. Bacterial inocula were prepared (after 3 weeks storage at −80 °C) by streaking isolated bacteria onto 1.5% nutrient agar in 100 × 15-mm petri plates and evaluating them for purity after 48 hours’ incubation at room temperature. Single cell colonies from each bacterial culture were transferred to sterile vials with 50 ml Nutrient Broth (Sigma Chemical Co., St. Louis, MO) and incubated by shaking for 36 hours. Cultures were transferred to sterile 50-ml tubes under aseptic conditions and centrifuged at 1,000 rpm for 10 minutes to obtain a bacterial pellet. Pellets were resuspended in 15 ml sterile phosphate buffer (40 mM Na₂HPO₄ and 25.7 mM KH₂PO₄ in sterile distilled water, pH 7.2). The final suspension absorbance was measured for each
bacterium by a spectrophotometer at 620 nm, and adjusted to an average population of $2.4 \times 10^7$ cells per ml.

Inoculum of *T. semipenetrans* was obtained from naturally infected field roots. Eggs, juveniles, and males were removed from root surfaces by hand-rubbing the roots in water. The nematode life stages were collected on 74/25-µm nested sieves. Nematodes were separated from soil and plant debris by sucrose centrifugation (Jenkins, 1964), surface sterilized with cupric sulfate (1,000 ppm) for 30 minutes, and rinsed with 5 exchanges (500-cm³ volume each) of sterile distilled water.

Zoospores of *P. nicotianae* were obtained by removing nutrient agar plugs from actively growing colonies of *P. nicotianae* (P-117). Plugs were placed into sterile 60 × 15-mm petri plates containing 10 ml of half-strength V-8 broth prepared by mixing 110 ml of clarified V-8 juice with 890 ml of water. Plates were incubated in the dark at room temperature for 4 days to promote mycelial growth, after which the V-8 was decanted and 10 ml of sterile distilled water was added and decanted twice. Plugs were then incubated in 10 ml sterile distilled water for 4 days in the light at room temperature to produce sporangia. Plates were refrigerated for 30 minutes and returned to room temperature to liberate zoospores. The zoospore suspensions were decanted after 45 minutes, combined, and quantified using a hemacytometer (American Optical Co., Buffalo, NY).

In both experiments, sour orange (*Citrus aurantium* L.) seeds, freshly extracted from fruit, were air-dried. Seed coats were removed, and seeds were surface sterilized for 10 minutes with 10% commercial bleach (0.6% NaOCl) containing 0.01% Tween-20, then rinsed five times in sterile distilled water. The laboratory experiment was conducted in 100-ml glass test tubes into which a single sterile decorticated seed was placed into the autoclaved soil mix, 50:50 by volume, Candler fine sand (uncoated, hyperthermic Typic Quartzipsamment sand) and shredded Canadian sphagnum peat moss (Scotts Inc., Sandusky, OH), and allowed to germinate and grow. The pH of the soil mix for half the plants was adjusted from 4.5 to 7.0 by addition of 3 ml per tube of 10% CaCO₃ to favor nematode infection. The experiment was conducted in natural light at a window at a temperature of 25 ± 2 °C. In the greenhouse experiment, sterile seedlings were germinated individually and aseptically in 150 × 25-mm autoclaved capped tubes containing MT medium (Murashige and Tucker, 1969) solidified with 0.9% agar (Difco, Detroit, MI) and containing 3% sucrose (pH 6.2). The germinating seeds were maintained in a growth room (26 °C ± 2) with continuous white fluorescent light. After 3 weeks seedlings were transferred to plastic Ray-Leach conetainers (3.8-cm diam. × 21-cm depth) (Stuewe & Sons, Inc, Corvallis, OR) filled with autoclaved soil mix (pH 4.5) as described previously. Thereafter, the experiment was maintained in the greenhouse (22 to 24 °C with 14 hours light per day).

In both experiments, suspensions (2 ml) of the appropriate bacteria treatments were pipetted onto the growth media immediately after decorticated seeds were placed there for germination. Two milliliters of sterile distilled water was added to media of seeds not receiving bacteria. Forty-five days after germination in the laboratory experiment and 10 days after transplanting the seedlings in the greenhouse experiment, a mixture of 90,000 eggs and second-stage juveniles of *T. semipenetrans* in 10 ml water was pipetted into the soil mix in four holes around each plant in treatments receiving nematodes. Control plants received 10 ml water. Nematodes were permitted to establish on the seedling roots for 6 months before *P. nicotianae* treatments were added. Zoospores of *P. nicotianae* (90,000) in 10 ml water were introduced via canula and distributed over the depth of 1 to 10 cm in soil of appropriate experimental units. To serve as a control, 10 ml water was added in the same manner to units not receiving zoospores.

Six weeks after fungal inoculation in both experiments, soil was rinsed gently from the plastic multipot cones or glass tubes to remove the plants. Roots were blotted and roots were separated as (tap or fibrous) and weighed separately. Stem fresh and dry weights were also measured. Fibrous roots from five plants per treatment were cut into 2-cm segments and mixed, and 5 g were processed (Duncan and El-Morshedy, 1996) to estimate the numbers of eggs, second-stage juveniles, and females per gram of root. Serial dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$) were made of the macerated root suspensions, and a 300-µl aliquot from each was plated onto nutrient agar to determine numbers of CFUs of bacteria. Bacterial isolates were identified as described previously. Roots from the remaining five replicates per treatment were washed free of soil with a minimum of tap water, dried for 48 hours in the oven (70 °C), and ground with a mortar and pestle. The concentration of *P. nicotianae* fungal protein in 30-mg samples of ground roots was determined using the Agri-screen Phytophthora detection immunoassay kit (Neogen Corp., Lansing, MI).

Subsets of the data from both experiments were analyzed by ANOVA of balanced completely factorial treatments. For laboratory experiments, three-way ANOVA was performed to test for the effects of pH, *P. nicotianae*, and either *B. megaterium*, *B. cepatia*, or *T. semipenetrans* on seedling root and shoot weight, ELISA absorbance values (*P. nicotianae* protein in roots), and final numbers of nematodes and CFUs of each bacterium. All possible interactions also were included in the model. The same method was used to analyze data from the greenhouse, except that two-way ANOVA was used because pH was not a variable in that experiment. The
population data were transformed (loge n+1) prior to ANOVA, but untransformed means are reported.

Results

Field survey: The number of bacterial CFUs from T. semipenetrans-infected roots was greater than that from uninfected roots in all groves surveyed (Tables 1, 2). For the two locations that were surveyed twice (Lake Alfred and Ona), numbers of CFUs were affected by survey date, groves, and type of root segment (P ≤ 0.001) (Table 1). There were no significant interactions between type of root segment and the other variables. In the second survey, conducted at three sites, B. megaterium and B. cepacia were the dominant bacterial species recovered from nematode-infected and uninfected roots. In each grove, B. megaterium was recovered from all sites and B. cepacia was recovered from all but one site. The number of B. megaterium CFUs were 1.4 to 2.2 times greater (0.079) on nematode-infected segments than on non-infected ones, and numbers for B. cepacia were 2.0 to 2.6 times greater (0.047) on nematode-infected segments (Table 2). Arthrobacter ilicis, Stenotrophomonas maltophilia, and Arcanobacterium haemolyticum were also recovered from both Tylenchulus-infected and uninfected roots in at least one site within each grove. The isolated fungal community was dominated by Fusarium solani in each of the three groves (Table 2). There was no effect (P = 0.190) of nematodes on numbers of F. solani propagules. Species in the genera Trichoderma, Verticillum, Phytophthora and Penicillium were also recovered from at least one sample from each of the groves.

Inhibition of P. nicotianae by the isolated bacteria: All isolated bacteria inhibited growth of P. nicotianae in vitro (P ≤ 0.05) on average by 30% compared to the control treatment (Fig. 1). There were no significant differences in degree of inhibition among bacterial species.

Bacteria and the interaction between T. semipenetrans and P. nicotianae in the laboratory: Seedlings grew less well in the laboratory experiment at soil pH 4.5 compared to pH 7.0 (Table 3; Figs. 2, 3). Root and stem fresh weights in the untreated control treatment were 22% and 40% higher, respectively, at pH 7.0 than at pH 4.5. Higher pH was also more favorable for population growth of the bacteria, nematode, and fungus. Total bacterial CFUs for B. cepacia and B. megaterium-only treatments were 75% and 82% more numerous at pH 7.0 than at pH 4.5 (Fig. 4). At pH 7.0, root infection by the nematode-only treatment was more than four-fold that at pH 4.5 (P ≤ 0.05). Mean ± standard error of female nematode/g root was 554.6 ± 110.9 at pH 7.0 and 121 ± 28.2 at pH 4.5. Corresponding means for offspring (eggs plus second-stage juveniles)/g root were 2,583.9 ± 502.9 and 182.5 ± 24. In plants infected by P. nicotianae only, fungal protein in roots was 72% greater (P ≤ 0.05) at pH 7.0 than at pH 4.5 (P ≤ 0.05) (Fig. 5).

Phytophthora nicotianae was the only microorganism that reduced seedling root and stem fresh weights at either pH compared to untreated controls (Table 3; Figs. 2, 3). Burkholderia cepacia by itself had no effect on stem fresh weights but increased root fresh weight by 20% compared to those of untreated controls (P ≤ 0.02) (Fig. 2). There were no interactions between this bacterium and P. nicotianae. Bacillus megaterium and P. nicotianae interacted (P ≤ 0.001) in their effects on seedlings (Table 3). Bacillus megaterium had no effect on stem (P ≤ 0.28) or root weight (P ≤ 0.10) in the absence of P. nicotianae, but the bacterium increased stem (P ≤ 0.002) and root weight (P ≤ 0.001) in seedlings infected by the fungus compared to the fungus-only treatment. Phytophthora nicotianae reduced the stem

Table 1. Total number of bacteria colony forming units† isolated from root segments infected and uninfected by Tylenchulus semipenetrans collected from two different locations.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Uninfected</td>
</tr>
<tr>
<td>Lake Alfred</td>
<td>21,615 ± 1,922</td>
<td>8,597 ± 611</td>
</tr>
<tr>
<td>Ona</td>
<td>2,390 ± 349</td>
<td>855 ± 104</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>28.09</td>
<td>0.001</td>
</tr>
<tr>
<td>Grove</td>
<td>115.02</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment††</td>
<td>24.03</td>
<td>0.001</td>
</tr>
<tr>
<td>Year × grove</td>
<td>23.00</td>
<td>0.001</td>
</tr>
<tr>
<td>Year × treatment</td>
<td>0.01</td>
<td>0.911</td>
</tr>
<tr>
<td>Grove × treatment</td>
<td>0.05</td>
<td>0.830</td>
</tr>
<tr>
<td>Year × grove × treatment</td>
<td>0.16</td>
<td>0.690</td>
</tr>
</tbody>
</table>

†Numbers are the untransformed mean ± standard error of five replications from each grove in each survey. Data were log transformed prior to ANOVA.
††Roots were either infected or uninfected by Tylenchulus semipenetrans.
Tylenchulus semipenetrans, in combination with *B. cepacia* or *B. megaterium* at pH 7.0, reduced the fungal protein in the roots by 79% and 93%, respectively, compared to the fungus-only treatment (Fig. 5). At pH 4.5, the nematode in combination with either bacterium completely suppressed detection of *P. nicotianae* protein in roots. In treatments that contained *P. nicotianae*, the nematode increased stem weight (*P* ≤ 0.001) and root weight (*P* ≤ 0.001) and *B. cepacia* increased root weight (*P* ≤ 0.007) but not stem weight (*P* ≤ 0.19). There were no interactions between the nematode and bacterium for root and stem weight (*P* = 0.87 and *P* = 0.97, respectively), suggesting an additive effect on root weight. The same analysis for *B. megaterium* showed that the bacterium interacted with the nematode for both stem (*P* ≤ 0.006) and root weight (*P* ≤ 0.002). The nematode increased stem weight (*P* ≤ 0.001) and root weights (*P* ≤ 0.001) in the absence of the bacterium, but not in its presence (*P* ≤ 0.29 and *P* = 0.18, respectively).

In plants infected by *P. nicotianae*, the addition of *T. semipenetrans* increased numbers of bacterial CFUs (*P* ≤ 0.003) at both pH levels, regardless of inoculation with *B. cepacia* or *B. megaterium* (Fig. 4). An interaction occurred with pH for effect of the bacterium on the nematode population. *B. cepacia* doubled mean numbers of nematode females (76 vs 178) and offspring (77 vs 156) (*P* ≤ 0.001 and 0.004, respectively) in plants treated with both *T. semipenetrans* and *P. nicotianae* at pH 4.5 compared to the nematode plus fungus treatment; however, *B. cepacia* reduced nematode offspring by 75% (*P* ≤ 0.04) and did not affect females (*P* ≤ 0.41) at pH 7.0. *Bacillus megaterium* increased mean numbers of females (320 vs. 532; *P* ≤ 0.01) and offspring (1,399 vs 1,690; *P* ≤ 0.02) in plants infected by *P. nicotianae* and *T. semipenetrans*, at both pH levels.

*Bacteria and the interaction between T. semipenetrans and P. nicotianae in the greenhouse: Burkholderia cepacia interacted with P. nicotianae* in terms of stem (*P* ≤ 0.07) and root (*P* ≤ 0.02) weights (Table 4). The fungus reduced the weight of the stems by 36% (*P* ≤ 0.003) and roots by

Table 2. Numbers of colony forming units of *Bacillus megaterium, Burkholderia cepacia,* and *Fusarium solani* isolated from root segments infected and uninfected by *Tylenchulus semipenetrans* collected from three different locations in 1999.

<table>
<thead>
<tr>
<th>Grove location</th>
<th><em>Bacillus megaterium</em></th>
<th><em>Burkholderia cepacia</em></th>
<th><em>Fusarium solani</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>Lake Alfred</td>
<td>13,279.6 ± 1,630.2</td>
<td>5,964.7 ± 797.5</td>
<td>4,744.4 ± 867.5</td>
</tr>
<tr>
<td>Ona</td>
<td>5,901.5 ± 481.5</td>
<td>4,081.5 ± 840.3</td>
<td>1,275 ± 100.2</td>
</tr>
<tr>
<td>Bartow</td>
<td>5,808 ± 651.5</td>
<td>1,410.0 ± 550.4</td>
<td>1,440.6 ± 196</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th></th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grove</td>
<td>2.73</td>
<td>0.089</td>
</tr>
<tr>
<td>Root type</td>
<td>3.41</td>
<td>0.079</td>
</tr>
<tr>
<td>Grove × root type</td>
<td>0.99</td>
<td>0.389</td>
</tr>
</tbody>
</table>

Numbers are the untransformed mean ± standard error of five replicates from each grove. Data were log transformed prior to ANOVA.

† Roots were either infected or uninfected by *Tylenchulus semipenetrans.*

weights by 23% (*P* ≤ 0.001) and root weight by 28% (*P* ≤ 0.001) in the absence of *B. megaterium,* but did not affect stem (*P* = 0.48) or root (*P* ≤ 0.18) weights in the presence of the bacterium. Despite the effects of *B. megaterium* and *B. cepacia* on the pathogenicity of *P. nicotianae,* neither bacterium affected the amount of fungal protein in the roots (Table 3; Fig. 5).

Infection by *T. semipenetrans* increased stem fresh weights (*P* ≤ 0.03) (Table 3; Fig. 3). There were interactions between the nematode and fungus in terms of root fresh weight (*P* ≤ 0.005) and fungus protein in roots (*P* ≤ 0.001). The fungus reduced stem weight (*P* ≤ 0.02) and root (*P* ≤ 0.002) weights in the absence of the nematode but had no effect on stem (*P* ≤ 0.97) or root weights (*P* ≤ 0.25) in the presence of the nematode. Similarly, *P. nicotianae* treatments resulted in increased fungal protein in roots (*P* ≤ 0.001), whereas fungal protein in the *T. semipenetrans* plus *P. nicotianae* treatment did not differ from that in the untreated control (*P* ≤ 0.618) (Fig. 5).

![Fig. 1](image)

**Fig. 1.** Effect of nematode-associated bacteria on *Phytophthora nicotianae* mycelial growth in vitro. Bars followed by a common letter are not significantly different according to Dunnett test (*P* ≤ 0.05). B. m = *Bacillus megaterium,* B. c = *Burkholderia cepacia,* A. h = *Arcanobacterium haemolyticum,* A. i = *Arthrobacter ilicis,* and S. m = *Stenotrophomonas maltophilia.*
39% \((P \leq 0.001)\) in the absence of \textit{B. cepacia}, but did not affect stem \((P \leq 0.75)\) or root \((P \leq 0.11)\) weights in the presence of the bacterium. Similarly, \textit{B. cepacia} increased stem \((36%; \ P \leq 0.02)\) and root weights \((39%; \ P \leq 0.03)\) in the presence of the fungus but not in its absence \((P \leq 0.95\) and 0.31, respectively). In contrast to the laboratory experiment, \textit{B. megaterium} had no effect on stem \((P \leq 0.603)\) weight. However, as in the laboratory experiment \textit{B. megaterium} interacted with \textit{P. nicotianae} with regard to root weight \((P \leq 0.013)\). The fungus reduced root weight by 38% \((P \leq 0.001)\) in the absence of \textit{B. megaterium} but did not affect root weight \((P \leq 0.23)\) in the presence of the bacterium. Similarly, \textit{B. megaterium} increased root weight by 39% \((P \leq 0.06)\) in the presence of the fungus but not in its absence \((P \leq 0.11)\). As in the laboratory, neither \textit{B. cepacia} nor \textit{B. megaterium} affected the amount of \textit{P. nicotianae} protein in roots (Table 4; Fig. 5).

Average density \((\pm\) standard error) of nematode females \(g/\)root was \(14.6 \pm 3.6\), and offspring \(g/\)root was \(5.8 \pm 3.01\) in the nematode-only treatment. There were significant interactions between \textit{T. semipenetrans} and \textit{P. nicotianae} with respect to root \((P \leq 0.048)\) and stem \((P \leq 0.06)\) fresh weights (Table 4). Stem and root weights in fungus-infected plants were increased by 65% \((P \leq 0.05)\) and 33% \((P \leq 0.0001)\), respectively, by the presence of \textit{T. semipenetrans} (Figs. 2, 3). \textit{Phytophthora nicotianae} protein in roots infected by both the fungus and nematode was less than half that in roots infected by only the fungus (Fig. 5), but in contrast to the laboratory experiment the effect was not significant (Table 4).

In plants inoculated with \textit{P. nicotianae} and \textit{B. cepacia}, \textit{T. semipenetrans} increased bacterial propagule numbers \((P \leq 0.005)\) (Fig. 4). There was no effect of \textit{B. cepacia} treatment on numbers of \textit{T. semipenetrans} females and offspring \((9\) vs. 8 and 34 vs. 34, respectively). Treatment with nematodes reduced the number of bacterial CFUs in seedlings treated with \textit{B. megaterium} and \textit{P. nicotianae} \((P \leq 0.06)\). In contrast to the laboratory experiment, \textit{B. megaterium} reduced nematode offspring \((from 6 to 4)\) in plants treated with \textit{P. nicotianae} and \textit{T. semipenetrans} \((P \leq 0.046)\) but did not affect the number of nematode females.

**Discussion**

\textit{Tylenchulus semipenetrans} altered the microbial community in the citrus rhizosphere by increasing propagule densities of bacteria and fungi in each of the three groves studied. All of the isolated bacteria suppressed growth of \textit{P. nicotianae} in vitro; however, in contrast to \textit{T. semipenetrans}, neither bacterium inhibited growth of the fungus when inoculated in whole-plant experiments. Nevertheless, the nematode and both of the selected bacteria generally increased the growth of citrus seedlings infected by \textit{P. nicotianae}. These results suggest that multiple mechanisms may attenuate virulence of \textit{P. nicotianae} in roots infected by \textit{T. semipenetrans}.

The positive effect of \textit{T. semipenetrans} and \textit{P. nicotianae} on population increase of rhizosphere microorganisms likely results from leakage of nutrients from fungus-
induced root lesions, nematode infection sites, or gelatinous egg masses. Our results agree with Weller (1986), who reported that densities of indigenous, gram-negative bacteria of *Pseudomonas* spp. were greater on roots infected by *Gaeumannomyces graminis var. tritici* than on healthy plants. Rovira and Wildermuth (1981) used electron microscopy to show that bacteria proliferate in fungus-induced lesions. Bergeson et al. (1970) showed a significant increase of *Fusarium* propagules in the rhizosphere of roots inoculated simultaneously with *M. javanica* and *F. oxysporum* f. sp. *lycopersici* compared to roots inoculated with only the fungus.

The dominant bacterial species isolated in these surveys, *B. megaterium* and *B. cepacia*, are well-documented biological control agents (Liu and Sinclair, 1993; Mao et al., 1998b; Schroth and Hancock, 1981; Zheng and Sinclair, 1996). Both species have been described as "plant growth promoting rhizobacteria" (PGPR; Schroth and Hancock, 1981) because of their ability to improve plant growth by aggressively colonizing roots and preempting the establishment of deleterious rhizosphere microorganisms (Suszlow and Schroth, 1982). *Bacillus megaterium* forms endospores that are resistant to unfavorable conditions. The bacterium is a good root colonizer and rhizosphere competitor, and remains viable for extended periods (Liu and Sinclair, 1993). Multiple effects of *B. megaterium* on soil microorganisms have been documented (Liu and Sinclair, 1993), and the bacterium has been shown to be a potential biocontrol agent for *Rhizoctonia* root rot of soybeans (Zheng and Sinclair, 1996). *Burkholderia cepacia* has been reported to colonize and enhance root hair development (De Freitas and Germida, 1990) and to produce a wide spectrum of antifungal metabolites (Lambert et al., 1987). The bacterium has been studied for biological control of diseases caused by many plant pathogenic fungi on different crops; examples include *R. solani*, *Pythium ultimum* (Mao et al., 1998a), and *F. oxysporum* (Larkin and Fravel, 1998) on tomato, damping-off diseases caused by species of *Pythium* and *Fusarium* (Mao et al., 1997, 1998b) on corn seedlings, and *F. oxysporum* f. sp. *lycopersici* on onion seedlings (Kawamoto and Lorbeer, 1976). PGPR also can alter plant physiology and increase the host plant defenses to pathogen attack (induced resistance). Colonization of roots by PGPR suppressed anthracnose of cucumber leaves.
caused by *Colletotrichum orbicular* (Wei et al., 1991). Root infection with *Pseudomonas* strain WC 5417r reduced the incidence of fusarium wilt in carnation and the amount of *F. oxysporium* f. sp. *dianthi* in plant stems (Van Peer et al., 1991).

The suppressive effect of *B. cepacia* and *B. megaterium* on growth of *P. nicotianae* in vitro is in agreement with Turney et al. (1992), who noted that all bacterial isolates collected from citrus rhizosphere soil inhibited growth of *P. nicotianae* on agar plates. However, no general relationship exists between the ability of a bacterium to inhibit a pathogen in vitro and suppress disease caused by the pathogen in vivo (Baker, 1987; Schroth and Hancock, 1981). Despite their potential as biological control agents, neither bacterium in this study showed evidence of suppressing the rate of infection or growth of *P. nicotianae* in citrus seedlings. Therefore, the results of the whole-plant experiments suggest that these bacteria may increase tolerance of citrus seedlings to infection by *P. nicotianae*. Both bacteria increased weights of citrus seedlings infected by *P. nicotianae*, and generally the fungus did not affect weights of the roots or stems of seedlings that were treated with either bacteria. We did not investigate causes of bacteria-induced seedling tolerance to *P. nicotianae*. Possible mechanisms by which PGPR can enhance plant tolerance are poorly understood, but may involve favorable modifications to the rhizosphere chemistry. *Bacillus megaterium* and other *Bacillus* spp., formulated commercially as Phosphobacterin (Copper, 1959; Mishustin, 1963), increased the vigor of wheat in the greenhouse but not in the field, possibly by transformation of unavailable minerals and organic compounds or by production of biologically active substrates such as auxins or gibberellins (Broadbent et al., 1977; Burr et al., 1978).

The effect of *T. semipenetrans* on growth of *P. nicotianae*-infected seedlings in a greenhouse was consistent with those in laboratory experiments previously reported (El-Borai et al., 2002b). The general lack of additive or synergistic effects by combinations of bacteria and nematodes is not surprising because treatment with either bacteria or nematodes tended to result in normal seedling growth similar to that of untreated controls. Moreover, these experiments were not controlled to the extent that involvement by these two bacteria in the effects demonstrated by the nematode treat-
ment can be discounted. All treatments with *T. seminpenetrans* increased microbial populations, and both bacteria were encountered in all treatments by the end of the experiment. However, unlike either bacterium, all treatments with nematodes reduced the amount of *P. nicotianae* protein detected in roots. This is the only direct evidence from these experiments for a possible mechanism by which pathogenicity of the fungus is attenuated by the presence of another organism. Eggs of *T. semipenetrans* were shown recently to be inhibitory to the growth of *P. nicotianae* (El-Borai et al., 2002a). Therefore, it is likely that infection of citrus roots by *T. semipenetrans* reduces population growth of *P. nicotianae* by direct antibiosis, which mitigates virulence of the fungus. All *T. semipenetrans*-induced changes in the microbial community revealed in this study appear to be favorable to the citrus root system; however, whether augmentation of natural levels of bacteria by nematodes has a significant effect on the plant is unknown.

The effects of *B. cepacia* on *T. semipenetrans* offspring at higher pH were consistent with Meyer et al. (2001), who showed that *B. cepacia* (Bc-2 and Bc-F) and *Trichoderma viride* (G1-3) suppressed numbers of root-knot nematode eggs and juveniles on roots of pepper plants. *Pseudomonas aureofaciens* inhibited *Criconema xenoplax* egg hatch in vitro and reduced nematode population densities in the greenhouse (Westcott and Kluepfel, 1993). The effect of *B. megaterium* on *T. semipenetrans* in the greenhouse experiment was consistent with Neipp and Becker (1999), who showed that two strains of *B. megaterium* inhibited hatching of *Heterodera schachtii* cysts in vitro and reduced nematode numbers and infection of sugarbeet seedlings when eggs were used as inoculum. However, the bacterium did not affect *H. schachtii* root infection in growth pouches. Treating potato plants with *B. megaterium* reduced population densities of *Meloidogyne chitwoodi* and *Pratylenchus neglectus* by up to 50% (Al-Rehiayani et al., 1999). Nevertheless, both bacteria increased numbers of *T. semipenetrans* under some of our experimental conditions. Differences in nematode species, or concomitant infection of the nematode with *P. nicotianae*, may account for variable effects of the bacteria on *T. semipenetrans* and other nematode species.

In conclusion, these studies indicate that infection of citrus roots by *T. semipenetrans* increases population densities of rhizosphere microorganisms, some of which may increase the tolerance of citrus seedlings to infection by *P. nicotianae*. However, we found no evidence that reduced infection of roots by the fungus in the presence of *T. semipenetrans* is mediated by changes in the rhizosphere microbial community. Additional studies that better control the combinations of nematodes, fungi, and bacteria, by excluding background contamination, are needed to demonstrate a direct effect of the nematode on suppression of root infection by the fungus. Field studies are also warranted to determine if disease caused by *P. nicotianae* is reduced by the nematode and whether the effect is important economically. Nevertheless, this study provides further evidence that *T. semipenetrans* is unlikely to exacerbate fibrous root rot of citrus caused by *P. nicotianae* (El-Borai et al., 2002a, 2002b).

### Literature Cited


