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Infection of Citrus Roots by *Tylenchulus semipenetrans* Reduces Root Infection by *Phytophthora nicotianae*[1]

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Abstract: Bioassays and whole-plant experiments were conducted to investigate the interaction between *Tylenchulus semipenetrans* and *Phytophthora nicotianae.* Both organisms are parasites of the citrus fibrous root cortex. Nematode-infected and non-infected root segments were excised from naturally infected field roots and placed on water agar in close proximity to agar plugs of *P. nicotianae* and then transferred to a *Phytophthora*-selective medium. At 10 and 12 days, 50% fewer nematode-infected segments were infected by *P. nicotianae* than non-infected segments. In whole-plant experiments in glass test tubes, sour orange seedlings were inoculated with two densities (8,000 or 80,000 eggs and second-stage juveniles) of *T. semipenetrans,* and after establishment of infection were inoculated with two densities (9,000 and 90,000 zoospores) of *P. nicotianae.* In the first experiment, fungal protein was 53% to 65% lower in the roots infected by both organisms than in roots infected by the fungus only. Compared to plants infected only by *P. nicotianae,* shoot weights were 33% to 50% greater ($P \leq 0.05$) in plants infected by both parasites, regardless of inoculum density. Fibrous and tap root weights were 5% to 23% and 19% to 34% greater ($P \leq 0.05$), respectively, in nematode-fungus combination treatments compared to the fungus alone. A second experiment was conducted, where plants were infected by the fungus, the nematode, both organisms, or neither organism. The soil mixture pH for 50% of the plants was adjusted from 4.5 to 7.0 to favor nematode infection. A higher rate of nematode infection of plants growing at pH 7.0 compared to pH 4.5 resulted in greater suppression of fungal development and greater inhibition of fungal damage to the plant. Compared to plants infected only by *P. nicotianae,* shoot and root weights were 37% and 33% greater ($P \leq 0.05$), respectively, in plants infected by both parasites. These experiments have revealed antagonism between *T. semipenetrans* and *P. nicotianae* in citrus.

Key words: citrus, competition, interspecific interactions, *Phytophthora nicotianae, Tylenchulus semipenetrans.*

Citrus is one of the most economically important crops in many areas with Mediterranean and subtropical climates. The most common association between nematodes and fungi pathogenic to citrus is that of *Tylenchulus semipenetrans* Cobb (1914) and *Phytophthora* spp. The citrus nematode, *T. semipenetrans,* is distributed throughout all citrus-growing regions of the world and causes the disease "slow decline," which results in significant reduction in fruit yield and size (Duncan and Cohn, 1990). The nematode is a semi-endoparasite of the cortical cells of citrus fibrous roots; the female induces several nurse cells in the root cortex, while the posterior part of the nematode remains exposed in the soil (Cohn, 1965; Van Gundy, 1958). The female is sessile and feeds from the nurse cells for its entire reproductive life. Citrus nematode eggs are deposited in a gelatinous matrix on the fibrous root surface.

Various species of *Phytophthora* also attack the citrus fibrous root cortex, causing a disease known as fibrous root rot (Graham and Menge, 1999), and the most common species in the subtropics is *P. nicotianae* Dastur Breda de Hann (synonym = *P. parasitica*) (Graham and Timmer, 1992; Hall, 1993). Fibrous root rot is characterized by soft, water-soaked lesions that expand and quickly result in cortex sloughing to leave only the threadlike vascular cylinder. Significant loss of fibrous roots due to infection by *P. nicotianae* can reduce fruit yield and size, similar to symptoms caused by *T. semipenetrans* (Graham and Menge, 1999).

A highly significant inverse relationship between numbers of nematodes following nematicide treatments and numbers of propagules of *P. nicotianae* in the soil was detected in field trials designed to evaluate nematicides and fungicides on concomitant populations of *P. nicotianae* and *T. semipenetrans* in a Florida citrus grove (Graham and Duncan, 1997). Because fibrous root density was not increased by nematode management, the inverse relationship suggested the possibility that the nematode may inhibit the fungus. We have subsequently investigated the nature of the interaction between *P. nicotianae* and *T. semipenetrans* in a series of field surveys and laboratory studies. In this paper, we report on experiments to determine whether infection of citrus fibrous roots by *T. semipenetrans* can modulate root infection by *P. nicotianae* and virulence of the fungus to citrus seedlings.

Materials and Methods

Phytophthora nicotianae infection of nematode-infected and non-infected root segments in vitro: A bioassay was conducted to determine whether *T. semipenetrans* infection of roots affects subsequent root infection by *P. nicotianae.* Nematode-infected citrus roots were collected from naturally infected trees and excised into segments (2 to 2.5 mm) that were either infected by a single female *T. semipenetrans* or not infected. Segments of each were surface-sterilized for 8 minutes in $CuSO_4$ (1,000 ppm) and then rinsed five times (500 cm^3 exchange of volume each time) in sterile distilled water.

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Phytophthora nicotianae isolate P-117 was cultured and maintained on *Phytophthora*-selective PARP-H medium (a cornmeal agar amended with antibiotics) (Graham, 1990; Mitchell and Kannwischer-Mitchell, 1992). Four agar plugs (5-mm diam.) were removed with a cork borer from the margins of actively growing *P. nicotianae* colonies and placed equidistant on 2% water agar medium in 100 × 15-mm petri dishes. Six nematode-infected root segments were placed 2 mm from each agar plug (24 segments per dish). Each egg mass, exposed on the root surface, was placed in contact with the water agar facing the fungus plug. The same procedure was repeated for root segments without the nematode. Each treatment was replicated six times. Root segments were of similar diameter and color. At 4, 7, and 11 days, root segments were removed from two dishes of each treatment (48 segments per treatment) and placed on PARP-H media for 72 hours to determine *P. nicotainae* infection. The experiment was repeated, but segments were evaluated after 12 days' exposure to *P. nicotianae*.

Effects of citrus nematode on fungal growth in roots and fungal virulence to citrus seedlings: Two whole-plant experiments were conducted to determine the effect of *T. semipenetrans* on the epidemiology of root infection by *P. nicotianae*. In both experiments, sour orange (*Citrus aurantium* L.) seeds were removed from fresh fruit, air-dried, and seed coats were removed. Seeds were surface-sterilized with 10% commercial bleach (NaOCl 0.6%) containing 0.01% Tween-20 for 10 minutes and rinsed five times in sterile distilled water. A single sterilized seed was placed in a 2-cm-deep depression made in the center of the surface of autoclaved soil mix (50: 50/v:v Candler fine sand (uncoated, hyperthermic Typic Quartzipsamments) and shredded Canadian sphagnum peat moss) (Scotts Inc., Sandusky, OH) in 150 × 25-mm glass test tubes. For the first experiment, seedlings were inoculated with two densities of the nematode and two densities of the fungus. Eight treatments included: fungus high, fungus low, nematode high, nematode low, fungus high with nematode high, fungus low with nematode high, fungus high with nematode low, fungus low with nematode low.

The second experiment was established in a similar manner. The pH of the soil mixture for 50% of the plants was adjusted from 4.5 to 7.0 by addition of 3 ml per tube of 10% $CaCO_3$, to favor nematode infection. Four treatments were established in a factorial design for each pH level in this experiment: (i) nematode-infected seedlings, (ii) fungus-infected seedlings, (iii) seedlings infected by both organisms, and (iv) seedlings infected by neither organism.

Both experiments were run with 10 single plant replicates per treatment in a completely randomized design arranged in racks in front of a window subject to natural lighting and maintained at room temperature (25 ± 2 ℃).

Inoculum of *T. semipenetrans* was obtained from naturally infected roots collected from the field. Eggs, juveniles, and males were collected on nested 74 and 25-µm pore sieves. Nematodes were separated from soil and plant debris by sucrose centrifugation (Jenkins, 1964), surface sterilized with $CuSO_4$ (1,000 ppm) for 30 minutes, and rinsed five times with sterile distilled water. In the first experiment, a 10-ml mixture of 8,000 or 80,000 eggs and second-stage juveniles of *T. semipenetrans* (low and high inoculum densities, respectively) were pipeted into four holes around the stems of each plant in treatments receiving nematodes. In the second experiment, 80,000 nematodes per plant were similarly inoculated. Nematode infection was established for 6 months before *P. nicotianae* was added.

Zoospores of *P. nicotianae* were obtained from colonies of isolate P-117 as described previously. Plugs were placed into sterile 60 × 15-mm petri plates containing 10 ml of sterile half-strength V-8 broth prepared by mixing 110 ml of clarified V-8 juice (Campbell Soup Co., Camden, NJ) with 890 ml of water, kept at room temperature in the dark for 4 days for mycelial growth, after which the V-8 juice 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 light at room temperature to produce sporangia. Plates were refrigerated 30 minutes and returned to room temperature to liberate zoospores. Zoospore suspensions were decanted after 45 minutes, combined, and quantified using a hemacytometer (American Optical Co., New York, NY). Low- and high-density (9,000 and 90,000 zoospores) fungal inocula in 10 ml water were introduced via canula, 1 to 10 cm deep, into the soil in the tubes in the first experiment. Only the high density of zoospores (90,000) was used in the second experiment. Ten milliliters of sterile distilled water was added in the same manner to control tubes.

Six weeks after fungal inoculation, soil was gently rinsed from tubes to remove the plants, roots were blotted, and tap and fibrous roots were separated and weighed. Stem and leaf fresh and dry weights were measured. Root systems from five plants per treatment were blender extracted (Duncan and El-Morshedy, 1996) to estimate the numbers of eggs, second-stage juveniles, and females per gram of root. Roots from the remaining replicates were gently rinsed with tap water and dried for 48 hours in an oven (70 ℃) and then ground by mortar and pestle. The concentration of *P. nicotianae* protein in 30 mg of dried roots was determined using the Agri-screen immunoassay kit (Enyzme Linked ImmunoSorbent Assay-ELISA) for detection of *Phytophthora* (Neogen Corp., Lansing, MI).

In the first experiment, the data from six balanced factorial treatments (fungus high, fungus low, fungus high nematode high, fungus low nematode high, fungus high nematode low, fungus low nematode low)

(all containing the fungus) were analyzed with 2-way ANOVA (Minitab Inc., State College, PA) in which fungus (high or low) and nematode (absent, high, or low) were main factors. Data in the second experiment were analyzed by 3-way ANOVA in which pH, fungus, and nematode were the main factors.

Results

Phytophthora nicotianae infection of nematode infected and non-infected root segments in vitro: In the first test, approximately twice as many root segments ($P \leq 0.01$) were infected by *P. nicotianae* in the absence of nematodes by 10 days' post-exposure (Fig. 1). Results of the second test at 12-day post-exposure were similar.

Effects of citrus nematode on fungal growth in roots and virulence to citrus seedlings: In two whole-plant experiments, infection by the citrus nematode reduced the fungal protein in the seedling roots and increased stem and root fresh weight compared to plants treated with only the fungus. In the first experiment (Fig. 2), levels of fungal protein in those roots infected by both organisms were 53% to 65% lower ($P \leq 0.05$) than in roots infected by only the fungus. Compared to plants infected only by *P. nicotianae,* shoot weights were 33% to 50% greater ($P \leq 0.05$) in plants infected by both parasites. Fibrous and tap root weights were 5% to 23% and 19% to 34% greater ($P \leq 0.05$), respectively, in nematode fungal combination treatments compared to the fungus alone (data not shown). There was no significant effect of *P. nicotianae* on *T. semipenetrans* reproduction. Mean (± standard error) nematode females/g root was 130 ± 26 in high inoculum treatments and 76 ± 10 in low inoculum treatments with corresponding offspring/g root = 1,953 ± 158 and 1,178 ± 198.

In the second experiment, soil pH affected the growth of seedlings and the population growth of the nematode and the fungus. Root and stem fresh weights of untreated controls were 22% and 40% greater, respectively, when grown at pH 7.0 compared to pH 4.5. The number of nematode females and offspring per gram of roots from plants inoculated only with nematodes were 3.6 and 13.2 times greater ($P \leq 0.05$) at high pH compared to low pH (Table 1). ELISA absorbance readings for *Phytophthora* sp. protein were 72% greater ($P \leq 0.05$) at pH 7.0 than at pH 4.5 for plants inoculated only with *P. nicotianae* (Fig. 3). *Phytophthora nicotianae* was the only parasite that reduced seedling mass at either pH. Compared to controls, *P. nicotianae* reduced root fresh weight ($P \leq 0.05$) by 37% at pH 7.0 and 16% at pH 4.5 (Fig. 3). The fungus reduced stem fresh weight ($P \leq 0.05$) by 27% at pH 7.0 and by 20% at pH 4.5 (Fig. 3).

Nematodes and fungi showed reciprocal inhibition in some conditions. *Tylenchulus semipenetrans* completely suppressed *P. nicotianae* protein in root tissue at both pH levels (Fig. 3). At pH 7.0, which favored nematode infection, the fungus had no effect on the final

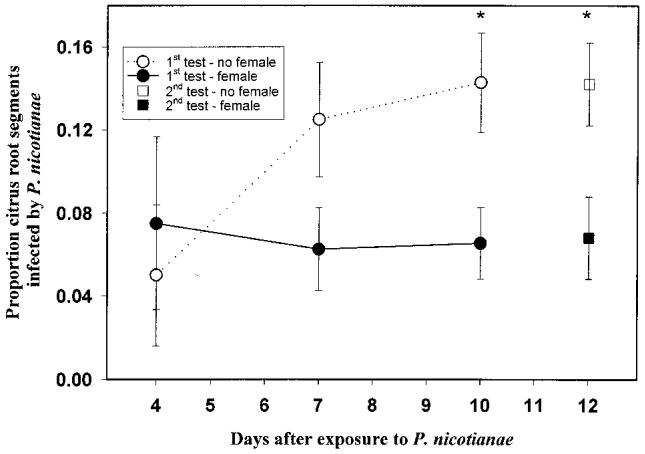

Fig 1. Infection of *Phytophthora nicotianae* on *Tylenchulus semipenetrans* infected and non-infected citrus root segments in vitro. Significant treatment effects (Student's *t*-test $P \leq 0.01$) denoted by asterisks.

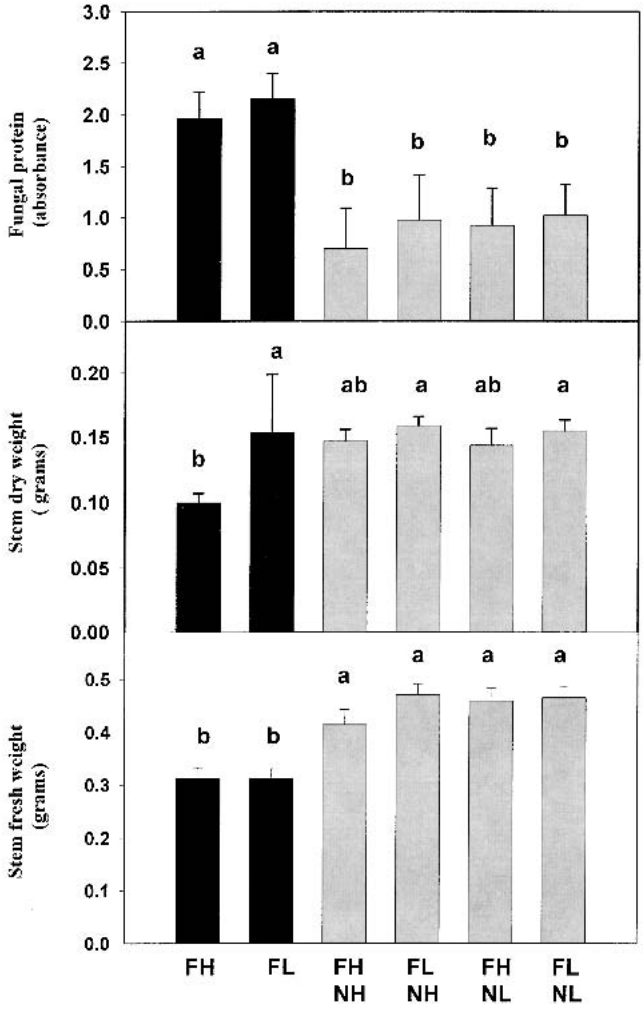

Fig 2. Effect of *Tylenchulus semipenetrans* on growth and pathogenicity of *Phytophthora nicotianae* (FH = fungus high density, FL = fungus low density, NH = nematode high density, and NL = nematode low density). Bars are standard error of the mean for 10 seedling replications per treatment. Bars with a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

TABLE 1. Effect of different soil pH on rate of reproduction of the citrus nematode *Tylenchulus semipenetrans*.

Treatment	Females g/of root		Offspring g/of root	
	pH 4.5	pH 7.0	pH 4.5	pH 7.0
Untreated control	—	—	—	—
T. semipenetrans	121.3 ± 38.2	554.6 ± 110.9	182.5 ± 24.0	2,583.9 ± 562.9
T. semipenetrans + *P. nicotianae*	76.4 ± 5.1	563.9 ± 152.3	77.7 ± 8.3	2,722.0 ± 688.2

Data are means of six replications ± standard error of the mean.

nematode density (Table 1). However, in plants grown at pH 4.5, which was less favorable for nematode infection, the fungus reduced ($P \leq 0.05$) the number of nematode females and offspring per gram of root by 39% and 58%, respectively (Table 1).

In seedlings, the presence of the nematode completely inhibited disease caused by *P. nicotianae* (Fig. 3). Regardless of soil pH, there were no significant differences in stem or root fresh weights in plants infected by both organisms compared to untreated controls (Fig. 3). The interaction between the nematode and the fungus was significant ($P \leq 0.05$) for fibrous root weight, and fungal protein.

DISCUSSION

An antagonistic interaction between *T. semipenetrans* and *P. nicotianae* in these experiments resulted in less

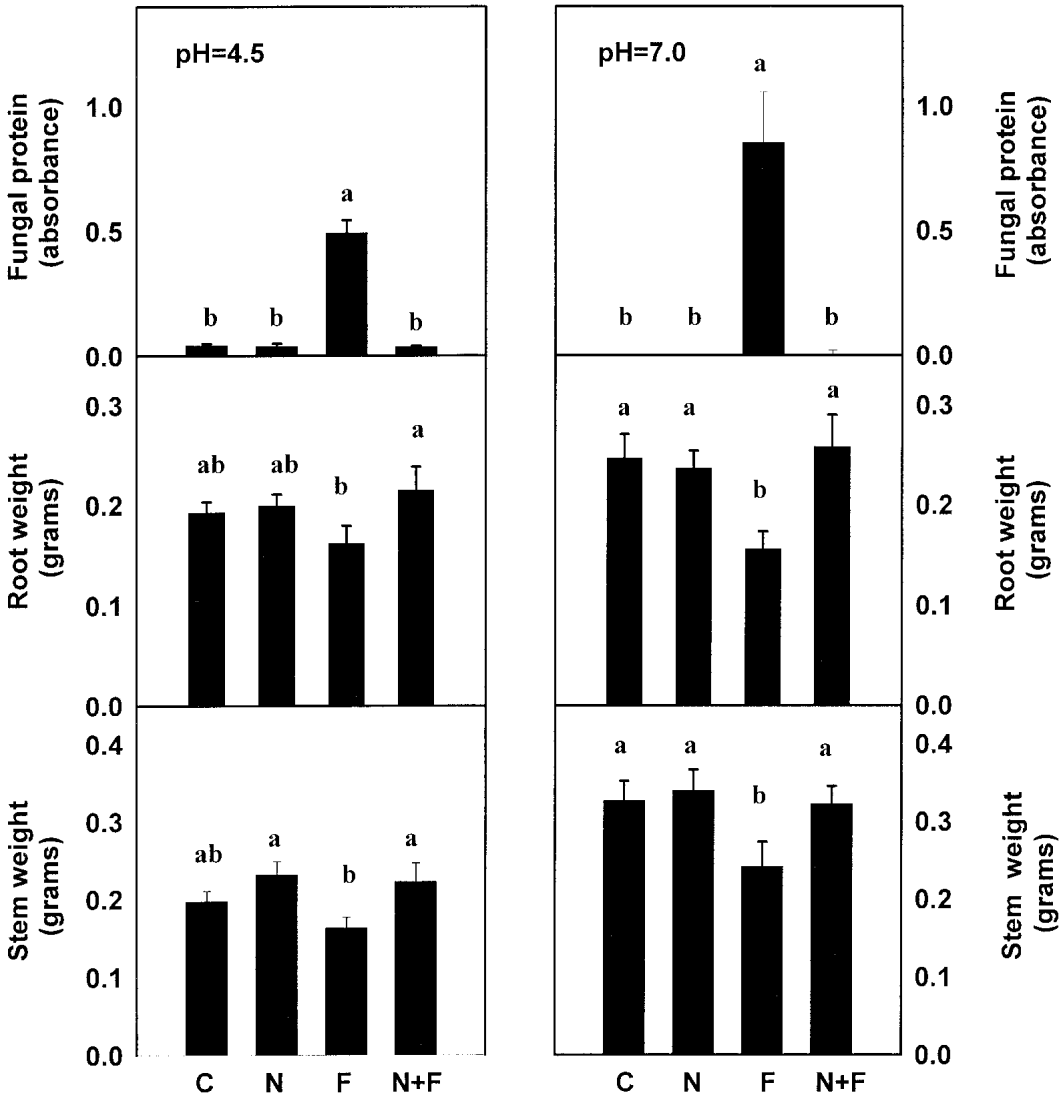

FIG 3. Effect of pH and *Tylenchulus semipenetrans* on growth in roots of *Phytophthora nicotianae* and damage by the fungus to citrus seedlings (C = control, N = nematode, F = fungus, N + F = nematode plus fungus). Bars are standard error of the mean for 10 seedling replications per treatment. Bars with a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

infection of roots by the fungus, reduced fungal development in roots, and less growth reduction of citrus seedlings. Although nematode densities among treatments were manipulated by different inoculation rates and different soil pH, no density dependence in the antagonistic effects was observed for the nematode densities in these experiments. The effect of pH is consistent with results of Van Gundy and Martin (1961), who found higher population densities of *T. semipenetrans* in alkaline than in acid soils. The pH optimum of soil for *T. semipenetrans* development is 6.0 to 7.5 (Van Gundy et al., 1964). The consistency of the antagonistic effect in these studies and observations of significant increases in *P. nicotianae* propagule densities in soil following management of *T. semipenetrans* in a citrus orchard (Graham and Duncan, 1997) suggest that the interaction may be of economic significance.

Nematode-fungal associations have been reviewed (Bergeson, 1972; Holdeman and Graham, 1954; MacGuidwin and Rouse, 1990; Mai and Abawi, 1987; Powell, 1971a, 1971b; Powelson and Rowe, 1993; Prot, 1993), and our results differ from those of most research involving the two groups of pathogens. When interactions occur, the nematode frequently plays the primary role as modifier of the host, making it more susceptible or suitable for other pathogens (Pitcher, 1978; Powell, 1979). Interrelationships between plant-parasitic nematodes and soil-inhabiting microorganisms were first observed by Atkinson (1892), who noted that the combination of *Meloidogyne* spp. and *Fusarium* wilt fungi in cotton contributed to more severe losses from wilt than did the fungus alone. Subsequently, root-knot and cyst nematodes were found to often predispose plants to heavier infection by other pathogens such as *Fusarium* spp., *Phytophothora* spp., and *Rhizoctonia* spp. (Carter, 1981; McLean and Lawrence, 1993; Powell et al., 1971; Roy et al., 1989; Webster, 1985; Whitney, 1974). The sting nematodes, *Belonolaimus* spp., ectoparasitic nematodes that cause little damage to the cortex of plants when feeding, have been associated with increased *Fusarium* wilt incidence in cotton (Yang et al., 1976). Synergistic disease complexes involving migratory endoparasitic nematodes include *Pratylenchus penetrans* and *Verticillium dahliae,* resulting in the potato early dying syndrome (Bowers et al., 1996), and *F. oxysporium* and *Pratylenchus penetrans* on alfalfa (Mouza and Webster, 1982).

Antagonism toward plant-pathogenic fungi by nematodes is not unknown (Costa Manso and Huang, 1986; Gray et al., 1990; Valle-Lamboy and Ayala, 1980). An interesting antagonistic interaction occurs between the reniform nematode (*Rotylenchulus reniformis*) and the cotton seedling blight fungus *Rhizoctonia solani* (Sankaralingam and McGawley, 1994). The presence of *R. solani* increased reproduction by *R. reniformis,* and the combined effect of the nematode and the fungus inhibited cotton seedling blight compared to plant inoculated with only the fungus. The similarity between results of the present study and that of Sankaralingam and McGawley (1994) is noteworthy because both *T. semipenetrans* and *R. reniformis* are sedentary semiendoparasites that infect roots by penetrating the mature epidermis and root cortex. This infection process is fundamentally different than that of numerous sedentary endoparasites that penetrate in the rapidly developing zone of elongation and for which antagonistic interactions with fungi are generally unknown.

Both *T. semipenetrans* and *P. nicotianae* are often found together in the citrus rhizosphere; therefore, it is not surprising that the nematode may protect its feeding site and so interfere with the fungus either indirectly (through resource competition, alteration of host physiology, or alteration of the microbial community in the rhizosphere) or directly (anti-fungal chemicals). *Tylenchulus semipenetrans* was shown to increase the incidence of *Bacillus megaterium* and *Burkholderia cepacia* in the citrus rhizosphere (El-Borai et al., 2000), and both bacteria inhibit a variety of soilborne plant pathogens (Al-Rehiayani et al., 1999; Mao et al., 1997, 1998; Millus and Rothrock, 1997; Zheng and Sinclair, 1999). Eggs of *T. semipenetrans* were recently found to inhibit mycelial growth of *P. nicotianae* and *F. solani* in vitro, in contrast to those of *M. arenaria* (El-Borai et al., 2002). Thus, the interaction between *T. semipenetrans* and *P. nicotianae* is probably complex.

Results of this study indicate that *T. semipenetrans* can reduce infection of citrus seedlings by *P. nicotianae* and mitigate disease caused by the fungus. It is unknown whether the nematode interacts in a similar way with *P. nicotianae* on citrus trees in the field. Field trials to examine this question are warranted due to the widespread occurrence of both pathogens in subtropical citrus orchards.

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