Induced Soil Suppressiveness to a Root-Knot Nematode Species by a Nematicide

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Investigations were conducted to determine whether the nematicide DiTera, could induce soil suppressiveness to the root-knot nematode *Meloidogyne incognita*. To determine the effects of DiTera on soil microorganisms with antagonistic potential to nematodes, DiTera was added to autoclaved and nonautoclaved soils, and at 10, 30, and 60 days after treatment (DAT), alginate screens with *M. incognita* eggs were buried in the soil for 70 h, removed, and incubated at 24°C for 5 days to determine percentages of parasitized eggs and juveniles. At 30 and 60 DAT, the nonautoclaved soil treated with DiTera had higher percentages of parasitized eggs and juveniles than did the control, whereas no effects of DiTera were observed in the autoclaved soil. To further investigate the effects of DiTera on soil microbial communities, populations of specific groups of microorganisms from the soil and rhizosphere of tomato plants were quantified after DiTera addition. An increase in the population level of several groups of microorganisms from the soil and rhizosphere after DiTera treatment was observed 30 and 60 DAT compared with the control. Changes in traits of microbial physiology that confer enhanced survival or antagonism toward nematodes were also investigated; however, the application of DiTera was not correlated with a change in frequency of any one specific physiological trait. To investigate functional changes of the microbial community, activities of several soil enzymes including fluorescein diacetate hydrolysis, catalase, chitobiase, protease, and urease were measured along with pH and electrical conductivity from autoclaved or nonautoclaved soil treated with DiTera at 0, 2.5, or 5.0 g/kg soil after 1, 10, or 43 DAT. More enzymatic activity was measured as early as 1 DAT in the treatments with DiTera compared with the control. Our results demonstrate that application of DiTera to soil led to enhanced antagonism of root-knot nematodes and that this was associated with structural and functional changes of the rhizosphere bacterial community. © 2001 Academic Press

INTRODUCTION

Since the 1950s, control of plant parasitic nematodes has relied on chemical nematicides. Due to the withdrawal of several nematicides because of environmental concerns, and due to the impending ban on methyl bromide as a soil fumigant, it has become important to find alternative nematode management options. Such alternatives include organic amendments (Godoy *et al.,* 1983; Rodríguez-Kábana *et al.,* 1987, 1992b; Kokalis-Burelle and Rodríguez-Kábana, 1994a,b; Walker *et al.,* 1998), cropping systems which use various antagonistic plants (Vicente and Acosta, 1987; Rodríguez-Kábana *et al.,* 1992a,c; Vargas-Ayala *et al.,* 2000), and introduced biological control agents (Becker *et al.,* 1988; Sikora, 1988; Oostendorp and Sikora, 1989; Spiegel *et al.,* 1991; Kluepfel *et al.,* 1993; Okan *et al.,* 1993; Zuckerman *et al.,* 1993; Hallmann *et al.,* 1997; Martínez-Ochoa *et al.,* 1997; Aalten *et al.,* 1998; Kempster, 1998). The main goal of all of these approaches is to induce soil suppressiveness to nematodes by increasing indigenous antagonists, introducing antagonists, or both.

Methods for demonstrating enhancement of antagonists and suppressiveness when nematode control is present have varied. General suppressiveness (Cook and Baker, 1983) involves enhanced populations or activity of general soil microorganisms, which can be detected by quantification of total populations of microorganisms or selected enzyme activities in soils. Specific suppressiveness or induced suppressiveness occurs through activity of a specific species or group of microorganisms (Cook and Baker, 1983), which can be detected by measurement of enhanced antagonistic activity (including direct parasitism of nematodes) due to a select group of microorganisms or by measurement of

specific changes in microbial community structure and physiology of soil or rhizosphere microorganisms.

Different approaches have been used to investigate the roles of soil or rhizosphere microorganisms in induced suppressiveness to plant-parasitic nematodes. General suppressiveness has been demonstrated after application of different amendments such as chitin or pine bark powder to the soil. Godoy *et al.* (1983) measured changes in total bacterial and fungal populations and some specific enzyme activities (nitrate–nitrogen, ammonia–nitrogen, and chitinase) in soil treated with chitin to control *Meloidogyne arenaria* (Neal) Chitwood in squash. Those authors observed the presence of a selected antagonistic mycoflora induced by the addition of chitin. Similarly, Kokalis-Burelle and Rodríguez-Kábana (1994b) reported that pine bark powder altered soil microflora, providing *Heterodera glycines* Ichinohe control, and they suggested that this control was correlated with increases in total fungal populations and trehalase activity of the soil.

Vargas-Ayala *et al.* (2000) reported that the use of velvetbean in crop rotations provided control of plant-parasitic nematodes by selecting a physiologically and taxonomically distinct microflora compared with rotations without velvetbean. Those authors reported that the induced suppressiveness resulting from velvetbean was not due to the action of one or a few specific antagonistic microorganisms as sometimes occurs with monoculture (Cook and Baker, 1983; Stirling, 1991), but they also could not demonstrate general suppressiveness as defined by Cook and Baker (1983), since total microbial populations did not increase.

Specific suppressiveness has been detected by quantification of changes in the population of a specific group of microorganisms in the rhizospheres of plants with antagonistic properties to plant-pathogenic nematodes by use of different selective media (Kloepper *et al.,* 1991). Plants with antagonistic properties had a distinct rhizosphere microflora, and although total rhizosphere bacterial populations from antagonistic and nonantagonistic plants were not different, there were differences in the physiological profiles of bacteria from antagonistic plants compared with those from nonantagonistic plants.

The contribution of microorganisms to induced suppressiveness can also be determined by analysis of the activity of soil enzymes associated with microbial activity. Activity of different soil enzymes has been correlated with microbial activity, microbial biomass, respiration, ATP content, reduction of certain pests and diseases, yield, and soil properties such as cation exchange capacity.

Fluorescein diacetate (FDA) hydrolysis has been reported as a simple, rapid, and sensitive method for the estimation of total microbial activity (Swisher and Carroll, 1980; Schnürer and Rosswall, 1982; Schnürer *et al.,* 1985; You and Sivasithamparam, 1994), although other methods such as respiration measurements, ATP analysis, and dehydrogenase activity can be used to assess it. FDA is hydrolyzed by several different enzymes, such as proteases, lipases, and esterases from active fungi, most bacteria, some protozoa, and algae.

Chitobiase activity has been correlated with increased numbers of fungal propagules (Rodríguez-Kábana *et al.,* 1983) and inversely correlated with number of *M. arenaria* galls/g of fresh squash root (Rodríguez-Kábana *et al.,* 1989). Chitin is a polymer common in nature as a constituent of structural tissues and cell walls of many organisms. Two hydrolases produced by specialized fungi and bacteria (chitinase and chitobiase) can hydrolyze chitin.

Catalase activity is related to bacterial and fungal counts, cation exchange capacity, cotton yield, and fertilization regime (Rodríguez-Kábana and Truelove, 1970, 1982; Markova *et al.,* 1990). Catalase is a characteristic enzyme for nearly all aerobic and facultative anaerobic microorganisms (Weigand *et al.,* 1995). Protection from activated oxygen species such as hydrogen peroxide can be achieve by enzymes such as catalase (Katsuwon and Anderson, 1990).

Nearly all microorganisms in soils are capable of protein degradation by protease enzymes. Proteases are present in living and active cells and in dead cells and are adsorbed to organic, inorganic, and organomineral particles. Under laboratory conditions, Asmar *et al.* (1992) demonstrated that the soluble fraction of extracellular protease was highly correlated with increases in total protease activity, total counts of bacteria, and ATP content in soil conditions promoting intensive microbial growth.

Ureases are a group of amidases that attack urea radicals. Additions of urea to soil resulted in reductions in number of galls produced by root-knot nematodes and an increase in numbers of microbivorous nematodes (Rodríguez-Kábana and King, 1980). Changes in urease activity were related to bacterial and fungal biomass determined microscopically (Nannipieri *et al.,* 1978).

We conducted the current study in an attempt to develop a more comprehensive approach to determining whether the induced suppressiveness observed after the addition of a nematicide was induced in that specific case by general or specific suppressiveness. To develop this approach, we selected a nematicide DiTera (Valent BioSciences Corp., Long Grove, IL) that is neither a typical chemical nor a biological control product, which in previous studies demonstrated nematode suppression. This product is obtained by submerged fermentation of the hyphomycete fungus *Myrothecium* spp. and is composed primarily of proteins and sugars. DiTera has been reported to kill plant-parasitic nematodes in the soil on contact (Grau *et al.,* 1997). In tests on free eggs, DiTera prevented eggshell permeability change (normally caused by host root exudates), im-

peding hatch of *Globodera pallida* (Stone) Behrens (Twomey *et al.,* 1998). We wanted to determine whether this product induced any change in the structure of the microbial community that could result in a microflora suppressive to nematodes. We report the use of four approaches—measurement of antagonistic potential, quantification of specific groups of aerobic soil bacteria, physiological activity of these bacteria, and soil enzyme activities—to investigate potential soil suppressiveness to nematodes following introduction of DiTera.

MATERIALS AND METHODS

Several experiments were designed to determine whether treatment of soil with DiTera caused structural or functional changes in the bacterial communities of the soil or the rhizosphere. The soil used in these experiments was a Pacolet fine sandy loam (thermic Typic Kanhapludults) collected from an experimental field on the Auburn University campus, which was selected because it was conducive to nematodes (Mitchell *et al.,* 1996). It was collected to a depth of 20 cm, sieved, and mixed 1:1 with sand (v/v). Half of the soil was autoclaved twice on consecutive days. Autoclaved and nonautoclaved soils were divided into 1-kg fractions, placed in polyethylene bags, and treated immediately with the levels of DiTera specified below.

Antagonistic Potential

To test the effects of DiTera on the antagonistic potential of soil toward nematodes, rates of 0, 2.5, or 5.0 g of DiTera/kg soil were used in autoclaved and nonautoclaved soil. After thorough mixing, soil was dispensed in 700-cm^3 plastic pots. Pots were placed in a randomized completed block design with six treatments (three concentrations of DiTera in two kinds of soil). For each treatment, eight replications and three sampling times (10, 30, and 60 days after treatment; DAT) were used. The experiment was conducted in a growth chamber under controlled-temperature conditions (28°C, 12 h light; 24°C, 12 h dark).

Cultures of *Meloidogyne incognita* (Kofoid & White) were maintained on the susceptible tomato cv. Rutgers for 3–4 months. Galled root systems were blended in 5.25% sodium hypoclorite solution for 3 min prior to collection of eggs with 100-, 325-, and 500-μm-mesh sieves.

Ten days after treatment of the soil with DiTera, all pots were inoculated with 350 $\pm$ 25 *M. incognita* eggs embedded in alginate screens (Rodríguez-Kábana *et al.,* 1994). Seventy hours later, the films were removed from the soil, rinsed with sterile distilled water, placed between moist paper towels in a petri dish, and incubated at 24°C. Five days later, numbers of parasitized, dead, and healthy eggs were counted for each alginate film with

methods modified from Kokalis-Burelle and Rodríguez-Kábana (1994b). Healthy eggs and juveniles were those with typical nematode morphology. Dead eggs were those that had all the egg content totally vacuolated or showed a deformed shell. Dead juveniles were those without a typical egg/second-stage juvenile (J2) morphology. Parasitized eggs and juveniles were those that developed a mycelium. Empty shell eggs were counted as hatched eggs. This process was repeated 1 and 2 months after DiTera was applied. Data were analyzed by two-way ANOVA in PC-JMP and means were separated at $P = 0.05$ with the Tukey–Kramer test.

Effect of DiTera on Populations of Specific Groups of Soil Microorganisms

The effects of DiTera on populations of some groups of soil bacteria and fungi were determined by use of the same rates of DiTera as those used in the previous test; however, only treatments with nonautoclaved soil were used. One and 2 months after DiTera treatment, 10 g of soil from each replication were mixed with 90 ml distilled water, shaken at 250 rpm for 20 min, serially diluted in phosphate buffer 0.02 M, and spiral-plated (Spiral Biotech, Bethesda, MD) on the following media: solidified 1/10th strength tryptic soy broth agar (TSBA) for total and heat-resistant bacteria (the first dilution of each sample was placed for 20 min at 80°C before dilution and plating to determine heat-resistant bacteria), crystal violet agar (CVA) for Gram-negative bacteria (Gould *et al.,* 1985), reduced arginine–starch–salts agar (RASS) for actinomycetes (Herron and Wellington, 1990), chitin agar (ChA) for chitinolytic bacteria (Rodríguez-Kábana *et al.,* 1983), and potato dextrose agar (PDA) for fungi. After autoclaving, 100-ppm cycloheximide and 50-ppm nystatin were added by filtering (0.45 μm) in all the media except the PDA medium, in which 50-ppm chloramphenicol and 50-ppm streptomycin sulfate were added. The experiment was repeated once.

The colonies growing on 10% TSBA and CVA media were counted with a laser counter with bacterial enumeration software (Spiral Biotech), whereas the microorganisms growing in RASS, ChA, or PDA media were counted manually. Results were transformed to log colony-forming units (cfu)/g of soil sample. Data were analyzed by two-way ANOVA in the PC-JMP program, and means were separated at $P = 0.05$ with the Tukey–Kramer test.

Physiological Profiles

To determine whether DiTera treatment affected the functional diversity of soil bacteria, bacterial isolates were collected from the preceding experiment. With the 1/10 TSBA plates (total bacteria), 12 colonies/replication (72 colonies/treatment) were selected at random and streaked for purity on TSBA medium 1 and 2

months after treatment with DiTera. After incubation for 2 days at 28°C, purified colonies were transferred to 96-well plates filled with 50 μL TSBA/well, and these plates were incubated at 28°C for 48 h. Each well was then overlaid with 70 μL TSB + 30% glycerol and shaken for 48 h at room temperature. The plates were then stored at -80°C. The isolates were individually characterized for various physiological traits which confer enhanced survival or antagonism toward nematodes. These traits included casein hydrolysis, production of chitinases, and production of siderophores; methods used were as previously reported (Kloepper *et al.*, 1991). The experiment was repeated once.

Populations of Specific Groups of Rhizosphere Microorganisms

DiTera was applied at 0 and 5 g/kg soil to nonautoclaved soil. Three-week-old tomato seedlings (*Lycopersicon esculentum* Mill. cv. Rutgers) were transplanted into 20-cm-diameter pots 9 days after application of DiTera into the soil mixture. There were six replications per treatment and two plants per replication. Pots were placed in a greenhouse in a completely randomized block design.

Rhizosphere populations were determined by the removal of soil from the pots and the taking of 2 g of tomato roots plus tightly adhering soil from each replication. Two grams of roots were placed (30 DAT) in 250-ml Erlenmeyer flasks containing 50 ml of sterile phosphate buffer (PPB) (0.02 M), shaken at 150 rpm for 30 min, and serially diluted in 0.02 M PPB. At 60 days after treatment, the same procedure was done except that 10 g of roots was placed in flasks with 100 ml of PPB. Serial 10-fold dilutions of each treatment were spiral-plated onto the same media as before. Colonies were counted and results transformed to log cfu/g of root.

Effects of DiTera on Soil Enzyme Activities, pH, and Electrical Conductivity

To determine effects of DiTera on functional activity of soil microorganisms, activities of selected soil enzymes along with pH and electrical conductivity were assessed. All experiments were repeated at least two times, with representative results being presented. The same treatments as those in the study on antagonistic potential were used for soil enzyme analyses. Three-week-old tomato seedlings were transplanted as before at 10 DAT. There were seven replications per treatment. Pots with 1.3 kg of soil mixture were placed in a greenhouse in a completely randomized block design. Soil from each pot (200–300 g) was removed 1, 10 (at planting time), and 43 DAT, air dried, and stored at 4°C until used to determine pH, electrical conductivity, and enzymatic activities.

Soil pH was measured with a suspension of 5 g airdried soil and 5 ml of deionized water. After pH determi-

nation, 5 ml water was added; the suspension was centrifuged at 4000g for 20 min, and the electrical conductivity of the supernatant was measured with a conductivity bridge (Model RC B 2; Industrial Instruments, Inc., Cedar Grove, NJ) fitted with a conductivity cell ($K = 1.0$).

To determine activity of FDA hydrolysis (EC 3.1.1.6), 2 g of soil were placed in 30-ml plastic cups along with 10 ml of sterile potassium phosphate (8.7 g K_2HPO_4 and 1.3 g KH_2PO_4 in 1 liter of distilled water). The reaction was started by the addition of 1 ml of a stock solution of fluorescein diacetate (1 mg/ml in acetone). The cups were covered with a tray and incubated at 37°C for 45 min. The reaction was stopped by the addition of 10 ml of isopropyl alcohol. The contents of the cups were centrifuged at 5000g for 20 min. The optical density of the supernatant was determined at 490 nm. A standard curve was prepared by dissolution of fluorescein (Na salt) in isopropyl alcohol and phosphate buffer (50:50; v/v) to have a range of fluorescein concentrations of 0–15 μg/ml. Controls with twice-autoclaved soil were included to account for nonenzymatic decomposition of fluorescein diacetate. Controls with soil and water and without soil were also included to account for background interference.

Catalase activity (EC 1.11.1.6) was measured by the placing of 5 g of soil in 30-ml plastic cups and the addition of 5 ml of 0.3% (v/v) H_2O_2 and 5 ml of water. After incubation at 28°C for 30 min, 5 ml of 3 N H_2SO_4 was added to stop any further enzyme reaction. A 5-ml aliquot of the clear supernatant was titrated with 0.02 N $KMnO_4$ to the equivalence point. One drop of 1% $MnSO_4$ was added in each sample before titration. Controls with twice-autoclaved soil, to account for nonenzymatic decomposition of the peroxide, and water instead of substrate, to account for water-soluble permanganate oxidizable material in the soil samples, were included.

Chitobiase activity (EC 3.2.1.14) was determined by the placing of 2 g of soil in 30-ml plastic cups and the addition of 2 ml of substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (1 mg/ml). After incubation at 37°C for 6 h, 10 ml of ethyl alcohol was added to each sample. One-milliliter fractions of the supernatant were pipetted into test tubes containing 10 ml of water, and color was developed by the addition of 1 ml of 0.2 N NaOH to each tube. Optical density was determined at 420 nm and compared with a standard curve prepared with p-nitrophenol (0–10 μg/ml in ethanol–water). Controls with soil and water and without soil were also included to account for background interference.

Protease activity (EC 3.4.X.X.) was checked by the placing of 2 g soil in 30-ml plastic cups and treatment with 2 ml of sodium caseinate 2% (v/w). After the cups were closed tightly with lids, the cups were incubated for 6 h at 37–40°C. The reaction was stopped by the addition of 10 ml of 7.5% trichloroacetic acid. The contents were centrifuged at 3200 rpm for 20 min. A 1-ml
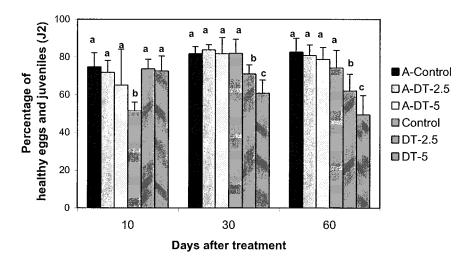
FIG. 1. Percentage of healthy *Meloidogyne incognita* eggs and juveniles (J2) from soil inoculated with alginate film 10, 30, and 60 days after treating (DAT) with DiTera (DT). A-Control, autoclaved control soil; A-DT-2.5, autoclaved soil treated with 2.5 g DiTera/kg soil; A-DT-5, autoclaved soil treated with 5 g DiTera/kg soil; Control, control soil; DT-2.5, soil treated with 2.5 g DiTera/kg soil; DT-5, soil treated with 5 g DiTera/kg soil. Data are means of eight replications. The bar at the top of each column represents the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each column for given days after treatment indicates nonsignificant differences.

aliquot of the clear supernatant was pipetted to a new plastic cup, and 6 ml of Laury reagent was added for 10 min. After that, 0.3 ml of water and Folin reagent (2:1) (v/v) were added and the cups were shaken. The optical density at 500 nm was read after 30 min. The standard curve was prepared with tyrosine. Control samples with autoclaved soil were included in the analysis.

Urease activity (EC 3.5.1.5; urea amidohydrolase) was measured by the placing of 2 g soil in 30-ml plastic cups and the addition of 2 ml 0.5 M urea. The cups were closed with tight lids and incubated for 3 h at 37°C. The reaction was stopped with 10 ml of a 10% NaCl solution (pH 2.52). The contents of the cups were centrifuged at 3200 rpm for 20 min. A 1-ml aliquot of the clear supernatant was pipetted to a new plastic cup, and 5 ml of an active reagent and 2 ml of 0.1% of

dichloroisocyanuric acid were added. After 30 min the optical density was measured at 690 nm. The standard curve was prepared with ammonium chloride. Control samples with autoclaved soil were included in the analysis. With this process we determined the amount of ammonium that was already in the samples plus the ammonium produced by the action of the urease enzyme decomposing the urea added (A). The other 2 g of soil were placed in 30-ml plastic cups, and 10 ml of 10% NaCl (pH 2.52) was added. The samples were kept at room temperature for 1.5 h. Then the same procedure as before was followed. With this process we determined the amount of ammonia initially present in the soil (B). The urease activity (C) was then calculated as C = A − B. The results were expressed as μg NH$_4$-N/ h × g soil.

TABLE 1

Populations of Specific Groups of Bacteria and Actinomycetes from the Soil (log cfu/g Soil) 30 and 60 Days after Addition of 0, 2.5, and 5 g of DiTera per kg of Soil[a]

Treatment[b]	Days after application of DiTera	Total bacteria	Heat-resistant bacteria	Gram-negative bacteria	Chitinolytic bacteria	Actinomycetes
Control	30	7.1 (1.0)[c] a	6.0 (0.2) b	4.9 (0.3) a	5.5 (0.1) a	—
DiTera-2.5	30	7.3 (0.1) a	6.8 (0.2) a	4.9 (0.5) a	5.2 (0.6) a	—
DiTera-5	30	7.3 (0.2) a	6.8 (0.2) a	4.9 (0.3) a	5.0 (0.3) a	—
Control	60	6.9 (0.1) b	6.1 (0.2) b	5.5 (0.2) a	5.8 (0.1) a	6.0 (0.1) a
DiTera-2.5	60	7.4 (0.1) a	7.3 (0.1) a	5.2 (0.5) a	4.9 (0.2) b	5.5 (0.2) b
DiTera-5	60	7.4 (0.2) a	7.3 (0.2) a	4.4 (0.3) b	4.5 (0.3) b	5.7 (0.2) b

[a] Nonautoclaved soil was used.
[b] Data are means of six replications. Statistical analysis was made separately for each sampling time. Means in the same columns followed by the same letter do not differ significantly according to the Tukey–Kramer test ($P \leq 0.05$).
[c] Value in parentheses represents the standard deviation of each average.

TABLE 2

Percentage of Soil Isolates Presenting Different Physiological Traits 30 and 60 Days
after Application of 0, 2.5, or 5 g DiTera/kg Soil

	30 days after treatment			60 days after treatment		
Treatment[a]	Chitin hydrolysis[b]	Casein hydrolysis[b]	Siderophore production[b,c]	Chitin hydrolysis[b]	Casein hydrolysis[b]	Siderophore production[b,c]
Control	4	79	78	3	68	25
DiTera-2.5	0	61	15	0	91	23
DiTera-5	0	100	52	2	100	81

[a] Each treatment consisted of 72 isolates selected at random from the plates with 1/10 TSBA, streaked twice in TSBA for purity, transferred to 96-well plates with TSA, flooded later with TSB + 30% glycerol, and frozen to −80°C until physiological traits were determined.
[b] (Positive reaction/positive reaction + negative reaction). Colonies not growing or presenting a weak growth were not counted.
[c] CAS medium is extremely toxic to the growth of Gram-positive bacteria.

RESULTS

Antagonistic Potential

DiTera induced antagonism to nematodes in nonautoclaved soil, as measured by the percentage of healthy nematode eggs and juveniles J2 (Fig. 1), the percentage of dead eggs (data not shown), or the percentage of eggs parasitized by fungi (data not shown) at 30 and 60 DAT. However, in autoclaved soil, addition of DiTera did not increase antagonistic microorganisms. At 10 DAT, the control without DiTera had significantly more parasitized eggs (i.e., less healthy eggs in Fig. 1) ($P < 0.05$) than the two DiTera rates. At 30 and 60 DAT, both rates of DiTera had significantly more parasitized eggs than the control, and the higher rate of DiTera resulted in significantly more parasitism than the lower rate ($P < 0.05$ at 30 DAT; $P < 0.05$ at 60 DAT).

Effect of DiTera on Populations of Specific Groups of Soil Microorganisms

Addition of DiTera to nonautoclaved soil resulted in significant changes in soil population densities of some groups of bacteria, with more changes occurring at 60 than at 30 DAT (Table 1). At 30 DAT, only populations of heat-resistant bacteria were significantly enhanced ($P < 0.05$) by DiTera treatment, whereas 60-DAT populations of total bacteria ($P < 0.05$) and heat-resistant bacteria ($P < 0.05$) were enhanced and chitinolytic bacteria ($P = 0.05$) and actinomycetes ($P = 0.05$) were reduced compared to the control. There were no differences in the population of Gram-negative bacteria in the lower rate of DiTera and the control, although a decrease was observed with the higher rate.

The most common genera of fungi found in the three treatments were *Penicillium* and *Aspergillus.* Different species of yeast were observed in the treatments with DiTera 60 DAT.

Physiological Profiles

Changes in traits of microbial physiology that confer enhanced survival or antagonism toward nematodes were investigated. An increase in the frequency of casein hydrolysis in the isolates selected from the DiTera soil was observed 60 DAT (Table 2). The frequency of

TABLE 3

Populations of Specific Groups of Bacteria and Actinomycetes from the Rhizosphere of Tomato Plants (log cfu/g Soil)
30 and 60 Days after Addition of 0 and 5 g of DiTera/kg of Soil[a]

Treatment[b]	Days after application of DiTera	Total bacteria	Heat-resistant bacteria	Gram-negative bacteria	Chitinolytic bacteria	Actinomycetes
Control	30	7.4 (0.2)[c] b	5.2 (0.3) b	7.0 (0.2) b	—	6.8 (0.3) a
DiTera-5	30	8.05 (0.1) a	7.1 (0.1) a	7.6 (0.1) a	—	6.7 (0.1) a
Control	60	7.6 (0.1) b	6.0 (0.3) b	7.2 (0.1) b	6.3 (0.2) a	7.0 (0.2) b
DiTera-5	60	8.0 (0.0) a	7.4 (0.1) a	7.3 (0.1) a	5.9 (0.2) b	7.4 (0.1) a

[a] Three-week old tomato seedlings cv. Rutgers were transplanted in each pot 9 days after application of DiTera into the soil mixture. Nonautoclaved soil was used.
[b] Data are means of six replications. Statistical analysis was made separately for each sampling time. Means in the same columns followed by the same letter do not differ significantly according to the Tukey–Kramer test ($P \leq 0.05$).
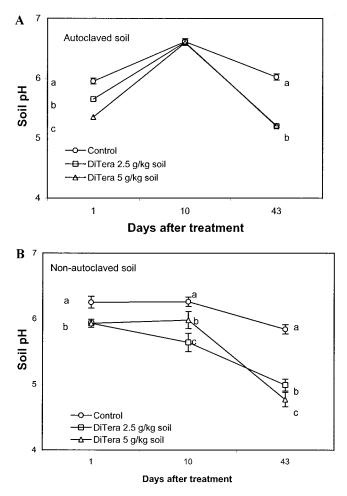[c] Value in parentheses represents the standard deviation of each average.

FIG. 2. Soil pH. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.

chitin hydrolysis was very low in the three treatments at both sampling times. No clear results were obtained after the testing of isolates from DiTera-treated and nontreated soil for siderophore production.

Populations of Specific Groups of Rhizosphere Microorganisms

In general, DiTera affected rhizosphere bacteria more than soil bacteria. Population densities of total bacteria, heat-resistant bacteria, and Gram-negative bacteria were higher in rhizospheres from the treatment with DiTera than from the control 30 DAT (Table 3). Chitinolytic bacteria population decreased by the addition of DiTera into the soil compared with that of the control, whereas populations of all the other groups were enhanced 60 DAT.

Effects of DiTera on Soil Enzymatic Activities

At the end of the experiment (43 DAT) the pH was more acid in the treatments with DiTera than in the

control, in both autoclaved and nonautoclaved soil (Figs. 2A and 2B). The electrical conductivity increased with DiTera in autoclaved and nonautoclaved soil, and that increase was proportional to the concentration of DiTera (Figs. 3A and 3B). At the end of the experiment, the differences between the treatments with DiTera and controls were smaller but were still significantly different ($P < 0.05$).

FDA hydrolysis increased in the nonautoclaved soil treated with DiTera 1, 10, and 43 DAT compared with the control (Fig. 4B), and this correlated with the increase in bacterial population detected from the soil and rhizosphere samples. There were no differences between treatments in the autoclaved soil 1 DAT (Fig. 4A), but at 10 and 43 DAT, an increase compared with the control occurred in the treatments with the nematicide in the autoclaved soils.

Catalase, protease, chitobiase, and urease activities increased in treated soil 1 day after application in the
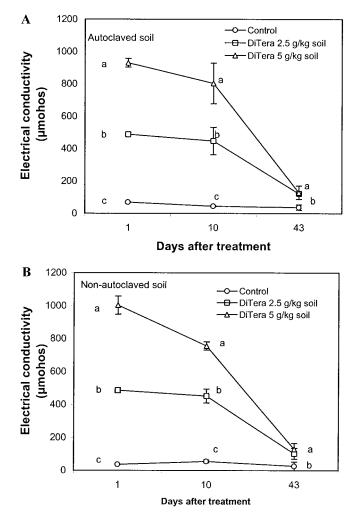

FIG. 3. Electrical conductivity. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.

A

B

FIG. 4. Fluorescein diacetate hydrolysis (FDA) in μg fluorescein/h × g of soil. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.
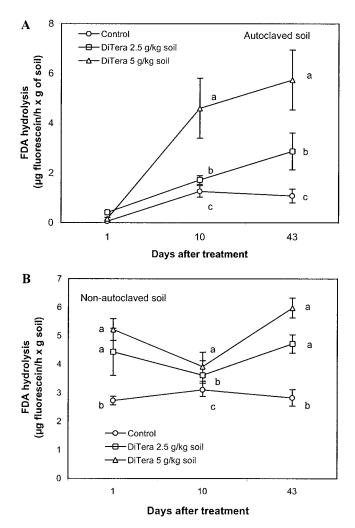
nonautoclaved soil, but at the end of the experiment, there were no significant differences among treatments (Figs. 5B, 6B, 7B, and 8B). In the autoclaved soil, treatments with DiTera also presented higher enzymatic activities than those of the control (Figs. 5A, 6A, 7A, and 8A).

DISCUSSION

The results reported here demonstrate that the use of the nematicide DiTera increased antagonism of soil, and this effect was maintained for the entire 2-month duration of the experiment (Fig. 1). This increased antagonism was likely the result of induced suppressiveness through the action of an altered microflora of the soil and rhizosphere; however, when discussing the potential role of general suppressiveness due to soil

microbial activity, it is impossible to prove a causal relationship that would exclude the potential involvement of other factors. For example, it could be argued here that DiTera persisted in the soil, thereby weakening the nematode eggs. Although we cannot totally exclude this as a contributing factor, we believe it is unlikely since DiTera is not a chemical nematicide and a precise nematicidal compound has not been detected, which supports the idea of an indirect mechanism of action through the stimulation of antagonistic microflora.

Application of DiTera caused changes in populations of some groups of soil microorganisms, and these changes were more evident in the soil samples 2 months after treatment than 1 month after treatment (Table 1). Interestingly, the effects of DiTera on population densities of bacteria were more pronounced in
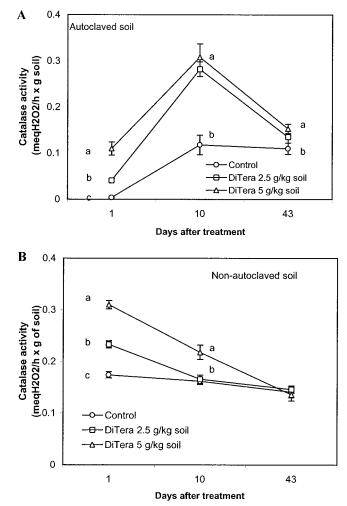
A

B

FIG. 5. Catalase activity in meqH$_2$O$_2$/h × g of soil. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.

FIG. 6. Chitobiase activity in μg *p*-nitrophenol/h $\times$ g of soil. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.
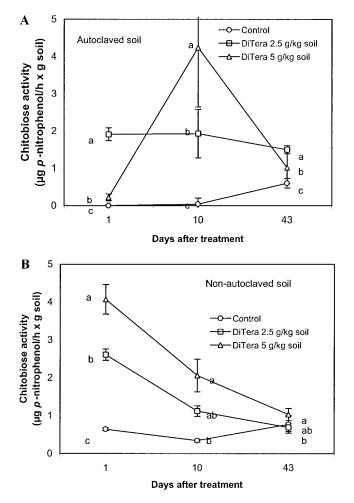
the rhizosphere than in the soil. For example, 30 DAT in the rhizosphere, DiTera induced increases in population densities of total, heat-resistant, and Gram-negative bacteria. However, 30 DAT in soil, DiTera induced a significant increase in population density only of heat-resistant bacteria. Therefore, even though soil populations were largely not affected by DiTera, there was an effect on the capacity of the bacteria to establish in the rhizosphere. These results suggest that the effect of soil microflora on rhizosphere populations should also be examined in experiments with organic amendments, rotation crops with antagonistic plants, or botanical aromatics.

Treatments with DiTera induced enhanced microbial physiological activity (based on enhanced enzyme activities) in the first few days after treatment. These enhanced enzyme activities were less evident later, but then the population changes in bacteria, which were

caused by DiTera, were still evident. The application of DiTera was associated with an increase of the frequency of the number of bacterial isolates that expressed proteolysis activity (casein hydrolysis), but no correlation with chitin hydrolysis or siderophore production was found.

Electrical conductivity was highly increased in the treatments with the product, and this increase was reduced with time, probably due to water percolation. The pH in the soil treated with DiTera was more acid than that in the control soil. Both the pH and the electrical conductivity were independent of the presence or absence of microorganisms since there were no differences between autoclaved and nonautoclaved soils and, hence, these changes are not indicative of induced suppressiveness but rather result from physicochemical properties of DiTera.

Differences in enzymatic activity between autoclaved and nonautoclaved soil were likely due to dif-
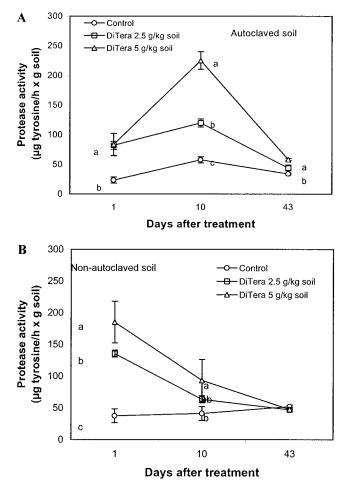

FIG. 7. Protease activity in μg tyrosine/h $\times$ g of soil. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.

FIG. 8. Urease activity in μg N-NH$_4^+$/h $\times$ g of soil. (A) Autoclaved soil; (B) nonautoclaved soil. Bars at each data point represent the standard deviation of the mean. Data were analyzed by the Tukey–Kramer test ($P \leq 0.05$). The same letter associated with each point for given days after treatment indicates nonsignificant differences.

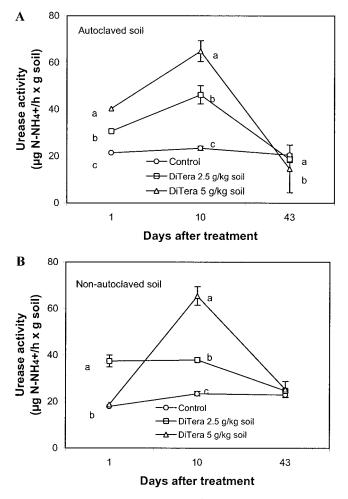In previous studies, shifts in specific functional groups of microbes detected by different methods have been used as suppressive disease indicators. These methods are (1) use of different selective media (Kloepper *et al.,* 1991); (2) determination of general microbial activities such as siderophore production, HCN production, chitin hydrolysis, casein hydrolysis and the ability to utilize a range of carbon substrates (Kloepper *et al.,* 1991; Pankhurst *et al.,* 1999); (3) measurement of enzyme activities such as FDA hydrolysis and trehalase activity (Godoy *et al.,* 1983; Kokalis-Burelle and Rodríguez-Kábana, 1994a); (4) determination of microbial diversity measured by fatty acid methy ester (FAME) profiles (Kloepper *et al.,* 1992; Pankhurst *et al.,* 1999) and organic matter stage of decomposition assessed by C:N ratio and cellulose and lignin content (Boehm *et al.,* 1993); and (5) a more recent alternative method based on the stability of nutrient cycles and energy flows after the introduction of stress to soil that uses the amplitude of waves of copiotrophic/oligotrophic bacteria and bacteria plus actinomycetes (van Bruggen and Semenov, 1999).

As these examples demonstrate, there are numerous experimental ways of providing evidence that changes in microbial activity or microbial diversity relate to supressiveness. However, the obtaining of direct evidence demonstrating that general or specific suppression has been induced is very difficult. For example, general or specific suppressiveness might have been induced, but the chosen experimental methods might not be sufficient to detect the underlying cause. If, for example, no changes are seen in activities of selected soil enzymes, the expected conclusion would be that general suppressiveness was not induced. Perhaps there was a significant undetected change in microbial physiology, which accounted for the suppressiveness. Similarly, false conclusions could be reached about the role of specific suppression based on the selected experimental methods. Therefore, there is not a unique method recommended for use as indicator of disease suppression; hence it is important to use many methods to have a better view of the mechanisms that are inducing suppression.

When multiple methods are used to examine the role of microbial communities in suppressiveness, it becomes less likely that "specific suppression" *sensu stricto* can be demonstrated, and we suggest that this term has very limited value. Even if a specific antagonist genus is found in suppressiveness, such as in induced suppressiveness to the cereal cyst nematode (Stirling, 1991), one cannot exclude the additional involvement of other microbial groups. Hence, a clear distinction between general and specific suppressiveness is not possible.

In our study, we used different approaches to assess the role of microorganisms in induced suppressiveness to nematodes via applications of DiTera. This nemati-

ferences in microbial populations between the soils. There was a general decrease in enzymatic activity from 1 to 43 DAT in the nonautoclaved soil, but this decrease was reached later in the autoclaved soil, in which the soil microflora needed time to recolonize the soil before manifesting higher enzymatic activity.

DiTera treatments presented higher FDA hydrolysis throughout the experiment, and this was correlated with the observed increase in soil and rhizosphere bacterial populations as reported in other studies (Schnürer and Rosswall, 1982; Schnürer *et al.,* 1985; Swisher and Carrol, 1980; You and Sivasithamparam, 1994). Lower FDA hydrolysis was detected at 10 DAT than at 1 DAT in DiTera treatments, although the same level of activity at 1 DAT was reached at 43 DAT. That could be explained by the fact that tomato plants were transplanted to those pots at 10 days after application of DiTera, and a reinoculation of each pot was performed due to the peat attached to the tomato roots.

cide apparently exhibits dual activity against nematodes by killing them directly on contact and by stimulating, over time, fungal antagonists and specific groups of bacteria from the soil and rhizosphere. We observed in our experiments quantitative changes in microbial functions (more soil enzymatic activity and an increase in proteolytic bacteria) and quantitative changes in the population levels of certain groups of microorganisms from the soil and rhizosphere (more antagonists, total bacteria, heat-resistant bacteria, Gram-negative bacteria, and actinomycetes). These results suggest that the induced suppressiveness caused by DiTera is not strictly of the general or specific types as defined by Cook and Baker (1983), and we suggest that these terms may be too restrictive to describe some cases of induced suppressiveness.

REFERENCES

Aalten, P. M., Vitour, D., Blanvillain, D., Gowen, S. R., and Sutra, L. 1998. Effect on rhizosphere fluorescent *Pseudomonas* strains on plant-parasitic nematodes *Radopholus similis* and *Meloidogyne* spp. *Appl. Microbiol.* **27,** 357–361.

Asmar, F., Eiland, F., and Nielsen, N. E. 1992. Interrelationship between extracellular enzyme activity, ATP content, total counts of bacteria and CO_2 evolution. *Biology and Fertility of Soils* **14,** 288–292.

Becker, J. O., Zavaleta-Mejía, 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.

Boehm, M. J., Madden, L. V., and Hoitink, H. A. J. 1993. Effect of organic matter decomposition level on bacteria species diversity and composition in relationship to *Pythium* damping-off severity. *Appl. Environ. Microbiol.* **59,** 4171–4179.

Canullo, G. H., Rodríguez-Kábana, R., and Kloepper, J. W. 1992. Changes in the populations of microorganisms associated with the application of soil amendments to control *Sclerotium rolfsii* Sacc. *Plant Soil* **144,** 59–66.

Cook, R. J., and Baker, K. F. 1983. "The Nature and Practice of Biological Control." APS Press, St. Paul, MN.

Godoy, G., Rodríguez-Kábana, R., Shelby, R., and Morgan-Jones, G. 1983. Chitin amendments for control of *Meloidogyne arenaria* in infested soil. II. Effects on microbial populations. *Nematropica* **13,** 63–74.

Gould, W. D., Hagedorn, C., Bardinelli, T. R., and Zablotowicz, R. M. 1985. New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. *Appl. Environ. Microbiol.* **49,** 28–32.

Grau, P., Rehberger, L., Hopkins, R., and Warrior, P. 1997. Development of DiTera—A biological nematicide. *J. Nematol.* **29,** 579. [Abstract]

Hallmann, J., Quadt-Hallmann, A., Rodríguez-Kábana, R., and Kloepper, J. W. 1997. Interactions between *Meloidogyne incognita* and endophytic bacteria in cotton and cucumber. *Soil Biol. Biochem.* **30,** 925–937.

Herron, P. R., and Wellington, E. M. H. 1990. New method for extraction of Streptomycete spores from soil and application to the study of lysogeny in sterile amended and nonsterile soil. *Appl. Environ. Microbiol.* **56,** 1406–1412.

Katsuwon, J., and Anderson, A. J. 1990. Catalase and superoxidismutase of root colonizing saprophytic fluorescent pseudomonads. *Appl. Environ. Microbiol.* **56,** 3576–3582.

Kempster, V. N. 1998. Induced resistance to nematodes? *Phytopathology* **88,** S46.

Kloepper, J. W., Rodríguez-Kábana, R., McInroy, J. A., and Collins, D. J. 1991. Analysis of populations and physiological characterization of microorganisms in rhizospheres of plants with antagonistic properties to phytopathogenic nematodes. *Plant Soil* **136,** 95–192.

Kloepper, J. W., Rodríguez-Kábana, R., McInroy, J. A., and Young, R. W. 1992. Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: Identification by fatty acid analysis and frequency of biological control activity. *Plant Soil* **139,** 75–84.

Kluepfel, D., McInnis, T. M., and Zehr, E. 1993. Involvement of root-colonizing bacteria in peach orchard soils suppressive of the nematode *Ciconemella xenoplax. Phytopathology* **83,** 1240–1245.

Kokalis-Burelle, N., and Rodríguez-Kábana, R. 1994a. Effects of pine bark extracts and pine bark powder on fungal pathogens, soil enzyme activity, and microbial populations. *Biol. Control* **4,** 269–276.

Kokalis-Burelle, N., and Rodríguez-Kábana, R. 1994b. Changes in populations of soil microorganisms, nematodes, and enzyme activity associated with application of powdered pine bark. *Plant Soil* **162,** 169–175.

Markova, N. L, Samsonova, A. S., and Zimenko, T. G. 1990. Effect of organic fertilizers on microflora of a polluted soil. *Pochvovedeniye* **4,** 73–80.

Martínez-Ochoa, N., Kloepper, J. W., Rodríguez-Kábana, R., and Pingsheng, J. 1997. Induced resistance and phenotypic characteristics of several PGPR compared to biocontrol activity against the root-knot nematode *Meloidogyne incognita. In* "Plant Growth-Promoting Rhizobacteria: Present Status and Future Prospects" (A. Ogoshi, K. Kobayashi, Y. Homman, F. Kodama, N. Kondo, and S. Akino, Eds.), Nakanishi Printing, Sapporo.

Mitchell, C. C., Arriaga, F. J., Entry, J. A., Novak, J. L., Goodman, W. R., Reeves, D. W., Runge M. W., and Traxler, G. J. 1996. "The Old Rotation: 1896–1996, 100 Years of Sustainable Cropping Research." Ala. Agric. Exp. Stn. Spec. Publ. Auburn University, AL.

Nannipieri, P., Johnson R. L., and Paul, E. A. 1978. Criteria for measurement of microbial growth and activity in soil. *Soil Biol. Biochem,* **10,** 223–229.

Okan, Y., Chet, I., and Spiegel, Y. 1993. Control of the root-knot nematode *Meloidogyne javanica* by *Bacillus cereus. Biocontrol Sci. Technol.* **3,** 115–126.

Oostendorp, M., and Sikora, R. A. 1989. Seed treatment with antagonistic rhizobacteria for the suppression of *Heterodera schachtii* early root infection of sugar beet. *Rev. Nematol.* **12,** 77–83.

Pankhurst, C. E., Hawke, B. G., and Magarey, R. C. 1999. Crop rotation-induced changes in the composition and functional diversity of soil microbial communities and its effect on yield decline in sugarcane. *In* "Proceedings of the 1st Australian Soilborne Disease Symposium" (R. C. Magarey, Ed.), pp. 5–8. Bureau of Sugar Experiment Stations, Brisbane.

Rodríguez-Kábana, R., and King, P. 1980. Use of mixtures of urea and blackstrap molasses for control of root-knot nematodes in soil. *Nematropica* **10,** 38–44.

Rodríguez-Kábana, R., and Truelove, B. 1970. The determination of soil catalase activity. *Enzimologia* **31,** 217–236.

Rodríguez-Kábana, R., and Truelove, B. 1982. Effects of crop rotation and fertilization on catalase activity in soil of the southeastern United States. *Plant Soil* **69,** 97–104.

Rodríguez-Kábana, R., Boube, D., and Young, R. W. 1989. Chitinous materials from blue crab for control of root-knot nematode. I. Effect of urea and enzymatic studies. *Nematropica* **19,** 53–74.

Rodríguez-Kábana, R., Godoy, G., Morgan-Jones, G., and Shelby, R. 1983. The determination of soil chitinase activity: Conditions for assay and ecological study. *Plant Soil* **75,** 95–106.

Rodríguez-Kábana, R., Kloepper, J. W., Robertson, D. G., and Wells, L. W. 1992a. Velvetbean for the management of root-knot and southern blight in peanut. *Nematropica* **22,** 75–80.

Rodríguez-Kábana, R., Kokalis-Burelle, N., Kiewnick, S., Schuster, R. P., and Sikora, R. A. 1994. Alginate films for delivery of root-knot nematode inoculum and evaluation of microbial interactions. *Plant Soil* **164,** 147–154.

Rodríguez-Kábana, R., Morgan-Jones, G., and Chet, I. 1987. Biological control of nematodes: Soil amendments and microbial antagonists. *Plant Soil* **100,** 237–247.

Rodríguez-Kábana, R., Morgan-Jones, G., Godoy, G., and Gintis, B. O. 1984. Effectiveness of *Gliocladium, Paecilomyces,* and *Verticillium* for control of *Meloidogyne arenaria* in field soil. *Nematropica* **14,** 155–170.

Rodríguez-Kábana, R., Pinochet, J., and Calvet, C. 1992b. El orujo de aceituna para el control de nematodos fitoparásitos. *Nematropica* **22,** 49–158.

Rodríguez-Kábana, R., Pinochet, J., Robertson, D. G., and Wells, L. 1992c. Crop rotation studies with velvetbean (*Mucuna deeringiana*) for the management of *Meloidogyne* spp. *J. Nematol.* **24,** 662–668.

Schnürer, J., and Rosswall, T. 1982. Fluoresceine acetate hydrolysis as a measure of total microbial activity in soil and litter. *Appl. Environ. Microbiol.* **43,** 1256–1261.

Schnürer, J., Clarholm, M., and Rosswall, T. 1985. Microbial biomass and activity in an agricultural soil with different organic matter contents. *Soil Biol. Biochem.* **17,** 611–618.

Sikora, R. A. 1988. Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. *Med. Fac. Landbouww. Rijksuniv. Gent* **53,** 867–878.

Spiegel, Y., Cohn, E., Galper, S., Sharon, E., and Chet, I. 1991. Evaluation of a newly isolated bacterium, *Pseudomonas chitinolytica* sp., nov., for controlling the root-knot nematode *Meloidogyne javanica. Biocontrol Sci. Technol.* **1,** 115–125.

Stirling, G. R. 1991. Natural occurring biological control. *In* "Biological Control of Plant Parasitic Nematodes: Progress, Problems and Prospects," pp. 99–124. CAB International, Wallingford, Oxon, UK.

Swisher, R., and Carroll, G. C. 1980. Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. *Microbial Ecol.* **6,** 217–226.

Twomey, U., Warrior, P., Kerry, B. R., and Perry, R. N. 2000. Effects of DiTera, a biological nematicide, on aspects of the life cycle of the potato cyst nematode *Globodera pallida. J. Nematol.* **12,** 355–362.

van Bruggen, A. H. C., and Semenov, A. M. 1999. A new approach to the search for indicators of root disease suppression. *Australasian Plant Pathol.* **28,** 4–10.

Vargas-Ayala, R., Rodríguez-Kábana, R., Morgan-Jones, G., McInroy, J. A., and Kloepper, J. W. 2000. Microbial shifts in soils and rhizosphere induced by velvetbean (*Mucuna deeringiana*) in cropping systems to control root-knot nematodes. *Biol. Control* **17,** 11–22.

Vicente, N. E., and Acosta, N. 1987. Effects of *Mucuna deeringiana* on *Meloidogyne incognita. Nematropica* **17,** 99–102.

Walker, J. T., Morris, B. D., and Melin, J. B. 1998. Nematicidal activity of plant tissue as soil amendments compared to DiTera®. *Phytopathology* **88,** S92.

Weigand, S., Auerswald, K., and Beck, T. 1995. Microbial biomass in agricultural topsoils after 6 years of bare fallow. *Biol. Fertil. Soils* **19,** 129–134.

You, M., and Sivasithamparam, K. 1994. Hydrolysis of fluorescein diacetate in an avocado plantation mulch suppressive to *Phytophtora cinnamomi* and its relationship with certain biotic and abiotic factors. *Soil Biol. Biochem.* **26,** 1355–1361.

Zuckerman, B. M., Dicklow, M. B., and Acosta, N. 1993. A strain of *Bacillus thuringiensis* for the control of plant-parasitic nematodes. *Biocontrol Sci. Technol.* **3,** 41–46.