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Effects of three microbial broth cultures and an organic amendment on growth and populations of free living and plant-parasitic nematodes on banana

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Abstract

The effect of 24 treatment combinations of cultures of *Streptomyces costaricanus* sp. nov. (ATCC55274), *Bacillus thuringiensis* (ATCC55273) and a strain of *Paecilomyces marquandii*, nematicide (cadusaphos), and/or wheat mash on growth and response of potted banana plants (*Musa* AAA) and populations of *Radopholus similis, Helicotylenchus multicinctus* and free living nematodes were studied in Río Frío, Costa Rica. The best plant responses (height, leaf numbers, healthy root weight), lowest numbers of plant parasitic nematodes and highest numbers of free living nematodes were observed for treatments containing wheat as a component. Two treatments, viz. wheat + *Streptomyces costaricanus* (200-ml culture) and wheat + *P. marquandii* (200-ml culture), gave the overall best results. Numbers of free living nematodes increased up to 1500-fold only for treatments containing wheat. Significant positive correlations existed between numbers of free living nematodes and shoot weight, healthy root biomass, plant height, and leaf numbers. Non-wheat treatments, including nematicide only, gave the poorest responses in general. Observations of nematodes sampled 50 days following planting in wheat-containing treatments showed most of the free-living nematodes ($\approx$ 90%) to be infected by nematophagous fungi (species not recorded). The results show that an organic amendment to soil, with or without a microbial component, can be an effective inducer of processes that regulate plant-parasitic nematode populations in soil.

Introduction

Banana (*Musa* AAA) is one of the most popular fruits in the world. Bananas amounted to 13.5 million metric tons in international trade in 1992, representing a 6-million ton increase in six years (Gowen and Quénéhervé, 1990; FAO, 1993). International banana trade accounts for only 12–15% of the total world production (Gowen and Quénéhervé, 1990). Plant-parasitic nematodes cause severe damage to bananas and significantly decrease their yield in most cropping systems as evidenced by increases in yield when nematicides are applied (Gowen, 1994; McSorley and Parrado, 1986; Sarah, 1989).

Chemical nematicide use is one of the primary means of control for plant-parasitic nematodes. However, the potential negative impact on the environment and ineffectiveness after prolonged use have led to a total ban or restricted use of most nematicides and an urgent need for safe and more effective options (Zuckerman and Esnard, 1994). Biological control promises to be such an option.

The efficacy of *B. thuringiensis* (Bt) strain CR-371rif$^+$ derived as a spontaneous rifampicin-resistant mutant of parent strain ATCC 55273 (Esnard, 1995), *S. costaricanus* sp. nov. Esnard, Potter and Zuckerman ATCC 55274 (Esnard et al., 1994; Esnard et al., 1995), and *P. marquandii* strain 3 for the suppression

of phytonematode populations has been demonstrated (Dicklow et al., 1993; Marban-Mendoza et al., 1992; Zuckerman et al., 1993; Zuckerman et al., 1989; Zuckerman et al., 1995). The rifampicin resistant mutant of Bt strain CR-371 (designated CR-371rif$^+$) was selected on solid media amended with rifampicin by the method of Liu and Sinclair (1992). CR-371rif$^+$ was used to study persistence in the soil. The biocontrol activity of the rif$^+$ strain and wild-type Bt have been compared in bioassays against the free-living nematode *Caenorhabditis elegans* (Esnard, 1995). The rif$^+$ Bt isolate had a significantly greater biocontrol effect on *C. elegans* than that of the wild type. The explanation for this is not known, but this observation and the antibiotic marker served as the basis for using the rif$^+$ Bt in this Costa Rica trial.

To assess the potential of certain antagonistic microorganisms and organic soil amendments for the management of plant parasitic nematodes (Sikora, 1992), we investigated the effect of a single application of certain combinations of 3 microbes, wheat mash and nematicide on banana and plant-parasitic and free living nematodes.

Materials and methods

Suspensions (0.3 ml) of the Bt strain (CR-371rif$^+$), previously stored at - 80 °C, were pipetted into 150-ml nutrient broth (NB) quantities and grown at ambient temperature (24 °C – 28 °C) for 48 h to $\approx 1 \times 10^8$ colony forming units (cfu)/ml, or for 120 h (to an undetermined cell density). Two inoculum levels were applied viz. 100 ml/pot (1X) and 200 ml/pot (2X).

Thawed suspensions (0.5 ml) of *S. costaricanus* were added to 150-ml volumes of potato dextrose broth (PDB). Cultures were grown for 120 h at room temperature to a final concentration of 10^5–10^6 cfu/ml. Inoculum levels applied to soil were 1X and 2X (as above).

Paecilomyces marquandii Strain 3 (0.5 ml) was also grown in 150 ml PDB with agitation (at 200 rpm) at room temperature for 120 h (to a final spore concentration of 1×10^6 cfu/ml). Cultures were applied at 1X and 2X volumes to the soil (as above). The 1X volumes of the bacteria or fungus were based on the lowest efficacious rates tested in previous greenhouse trials in Massachusetts (unpublished results).

Ground wheat was thoroughly mixed into the potted soil at 10 g/L soil and left undisturbed for 3 weeks before other treatment components were added (to allow for adequate breakdown of toxic products generated by decomposition of the wheat). Wheat, rice and a barley-wheat combination were previously tested in greenhouse and field trials in Puerto Rico (unpublished results). Wheat was chosen for the Costa Rica trial because it was readily available, less expensive than most nematicides used in banana production, and not associated with phytotoxicity 3 days after incorporation in the soil. The organophosphate nematicide Rugby® 10G (active ingredient, cadusaphos (FMC Corp.) which is structurally similar to ethoprop) was applied at full rate (10 g/pot) or $^1/_2$ rate (5 g/pot).

Large plastic pots (24-L, diam. 50 cm) contained 20 L of soil (with the following characteristics: 37.1% sand, 49.5% silt, 13.4% clay, pH 5.6, CEC 21.8 Meq/100 g, % base saturation K = 2.8, Mg 12.3, Ca 38.5; NO_3^--N = 46 ppm, $NH4^+$-N = 7 ppm, P = 8 ppm, K = 202 ppm, Ca = 1458 ppm, Mg = 284 ppm, extractable A1 = 138 ppm for soil range of 10–250 ppm; where K, Ca and Mg levels are very high, P at medium level, Pb low and micronutrient levels all normal (according to the Soil Testing Lab of the University of Massachusetts, Amherst, MA)). The soil originated in a naturally-infested banana field in Río Frío, Costa Rica where *Radopholus similis* and *Helicotylenchus multicinctus* are endemic.

Treatment components were applied 3 d (Bt, *Streptomyces* and nematicide), 7 d (*Paecilomyces*) or 21 d (wheat mash) before planting ≈ 5-cm tall tissue cultured bananas (Table 1). Nematode-infested untreated soil served as the control. The pots were maintained on wooden platforms (4 inches tall) on a concrete floor in a greenhouse in Río Frío, Costa Rica. Ambient temperatures ranged from 24 °C to 32 °C. All soil was watered simultaneously as needed. Soil and roots were sampled for nematodes 4, 50, and 92 days after planting. Nematodes were extracted from soil by the centrifugal sugar flotation method described by Barker and Niblack (1990). Nematodes in 25 g of macerated roots were screened with a 60-mesh sieve over a 325-mesh sieve.

At each soil sampling date, subsamples were also taken to analyze for persistence of the Bt CR-371rif$^+$ strain. Triplicate 50-g soil samples were taken from the pots containing treatments 8 and 13, and from untreated pots. The composition of each treatment is listed in Table 1. Thirty grams of each soil sample were stirred with 20 ml tap water. The suspension was passed through 4 nested sieves (pore sizes 425 μm, 75 μm, 45 μm and 25 μm = 500 mesh). Five ml of buffer (prepared by adding 10 ml of 1 M NaCl, 5 ml

Table 1. Description of treatment per pot. No., treatment number

No.	Treatments with wheat	No.	Treatments without wheat
1	Wheat	16	Untreated control
2	Wheat + 2X *Bacillus*	17	2X *Bacillus*
3	Wheat + 2X *Streptomyces*	18	2X *Streptomyces*
4	Wheat + 2X *Paecilomyces*	19	2X *Paecilomyces*
5	Wheat + *Paecilomyces* + *Streptomyces*	20	*Paecilomyces* + *Streptomyces*
6	Wheat + *Bacillus* + *Streptomyces*	21	*Bacillus* + *Streptomyces*
7	Wheat + *Streptomyces*		–
8	Wheat + *Paecilomyces*		–
9	Wheat + 5 g Rugby® 10 G/pot		–
10	Wheat + *Paecilomyces* + 2X *Bacillus*		–
11	Wheat + *Paecilomyces* + 2X *Bacillus*		–
12	Wheat + *Paecilomyces* + 120-h *Bacillus*		–
13	Wheat + *Paecilomyces* + 2X *Streptomyces*		–
14	Wheat + *Paecilomyces* + 2X *Streptomyces*		–
15	Wheat + *Bacillus* + 2X *Streptomyces*		–
		22	*Bacillus* + *Paecilomyces*
		23	*Bacillus* + *Streptomyces* + *Paecilomyces*
		24	10 g Rugby® 10 G/pot

– No corresponding treatment lacking wheat was tested. Treatments were replicated 5 times. Each pot contained 20 L of soil. Ground wheat was mixed into the soil (at 10 g/L soil) 3 weeks before application of other treatment components. *Bacillus* (*B. thuringiensis*) was a 48-h culture ($\approx 1 \times 10^8$ cfu/ml) applied at 100 ml/pot (1X), 200 ml/pot (2X), or a 120-h culture (of undetermined cell density) applied at 200 ml/pot. *Streptomyces* (*S. costaricanus*) was a 120-h culture ($\approx 10^5 - 10^6$ cfu/ml) applied at 100 ml/pot (1X), or 200 ml/pot (2X). *Paecilomyces* (*P. marquandii*) was a 120-h culture ($\approx 1 \times 10^6$ cfu/ml) applied at 100 ml/pot (1X), or 200 ml/pot (2X). Active ingredient in Rugby® 10 G is cadusaphos.

of 1 M phosphate buffer, 30 ml glycerol per 100 ml, and after autoclaving, 0.3 ml 0.1 M $MgSO_4$) were added. The suspensions were centrifuged at low speed for 2 min. A dilution series (to 10^{-6}) was prepared and 100 μl of each dilution were spread in triplicate on rifampicin (100 μg/ml) + cycloheximide (50 μg/ml)-amended nutrient agar in petri plates. Counts (cfu) were made after 24 h of incubation in the dark at 25 °C. Plants were harvested for final data recording from day 90 to 92.

The experiment consisted of 120 pots comprising 24 treatments (Table 1) with each treatment replicated 5 times and arranged in a completely randomized design. The Río Frío experiment was not repeated. Data were analyzed by ANOVA and means separated by Duncan's Multiple range test using MSTAT-C software version 1.4.

The following data on 17 variables were recorded for each plant: above-ground wet weight (g), number of leaves, height (cm), healthy root weight (g), dead root weight (g), lesion index (on a scale of 1–10), number of *R. similis*/25 g roots, number of *H. multicinctus*/25 g roots at day 4, 50 and 92 of planting, and numbers of *R. similis*, *H. multicinctus*, and free living nematodes per 100 g of soil on day 4, 50, and 92 of planting. Significant correlations between these variables were identified by generating Spearman rank correlation coefficients for all variable-to-variable comparisons. Only significant correlations are presented below. *Meloidogyne* spp. were observed in very small numbers in only two out of 120 samples. The nematode population data recorded are counts for subsamples of roots and soil.

Results

In general, the treatments associated with higher yields (Figure 1A), better plant responses (Figures 1A, B), low plant-parasitic (Figures 2A–C) and distinctively higher free living nematode populations levels than the untreated control (treatment # 16), microbe-only (treatments # 17 to # 23) and nematicide-only (# 24) treatments (Figure 2D) were those containing wheat mash as a component (treatments # 1 to # 15). Wheat mash in combination with the 200-ml culture of the

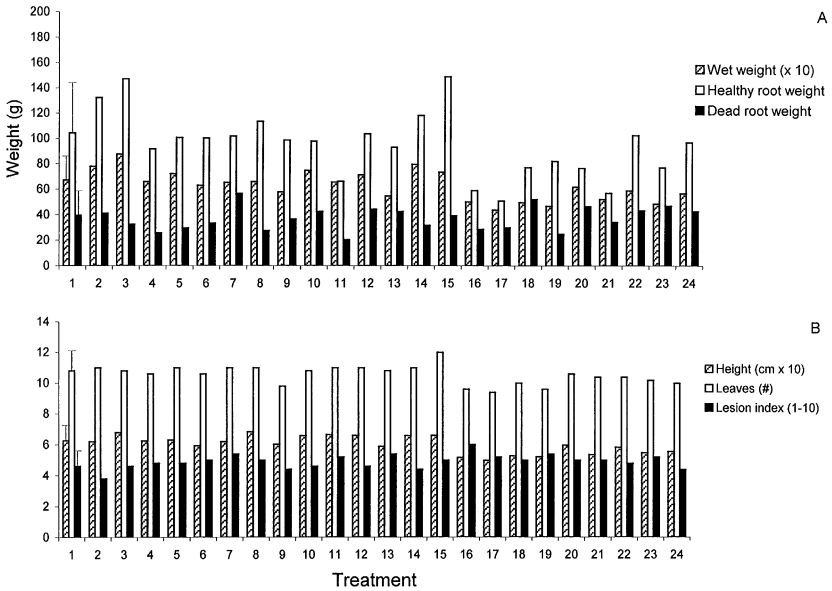

Figure 1. Response of banana plants to 24 treatments. (A) Shoot wet weight, healthy and dead root weight. (B) Plant height, number of leaves, and root lesion index. Treatments # 1 to 15 contain wheat, # 16 is the untreated control, and # 17 to 24 do not contain wheat (see key to treatments 1 to 24 in Table 1). The total length of each bar (┬) represents least significant difference ($p \geq 0.05$) within a series.

novel *Streptomyces* species (treatment # 3) gave the highest shoot weight and best responses overall (Figures 1, 2).

Analysis of the data matrix over 17 variables showed similarity in overall responses between the best treatments (viz. treatments # 1, 2, 3, 4 which contained wheat alone and wheat in combination with the higher application rate of *B. thuringiensis*, *S. costaricanus* or *P. marquandii* respectively). Non-wheat-containing treatments (viz. treatments # 16, 17, 18, 19, 20, 21, 22, 23, 24) coincided with the poorest responses when all variables were considered (Figures 1, 2). The nematicide-only treatment (# 24) also ranked among the poorest responses. The remaining treatments contained wheat (including the wheat plus nematicide treatment) and gave intermediate responses.

To measure correspondence between treatment ranks, Spearman rank correlation coefficients were generated for all pairs of variables. Apart from the expected high correlations between weight, leaf numbers, healthy biomass and plant height, population levels of free living nematodes correlated significantly (positively) with these variables (Table 2). Populations of free living nematodes increased dramatically (up to $\geq$ 1500-fold) only for applications of wheat-containing treatments (Figure 2D), even for the nematicide plus wheat amendment (viz. treatment # 9).

Table 2. Correlation (r_s) between free living nematode numbers in soil and wet weight of banana plant above soil line, number of leaves, height, and, healthy root weight

Free living nematodes	Wet weight	Leaves (#)	Height	Healthy roots
Day 4	0.61	0.55	0.71	0.42
Day 50	0.73	0.70	0.68	0.70
Day 92	0.73	0.53	0.70	0.66

Correlations significant at $p \leq 0.05$. Each datum is for 24 pairs of observations (replicated 5 times).

A high incidence of free living nematodes colonized by nematophagous fungi occurred in wheat containing treatments. Approximately 90% of all nematodes (and $\approx$ 10% of all stylet-bearing nematodes) observed in wheat containing treatments were infected. Abundant infections by *Arthrobotrys* species and some oomycetes were observed. Infected nematode species were not identified, and infected *Radopholus* and *Helicotylenchus* species were not observed in the soil samples collected.

The initial buildup of free-living nematodes associated with the wheat-only and other wheat-containing treatments persisted for 4 to 12 weeks (Figure 2D). By day 92, the effect of the wheat component decreased significantly. Where wheat and nematicide were added in tandem (treatment # 9), by day 50 (after planting)

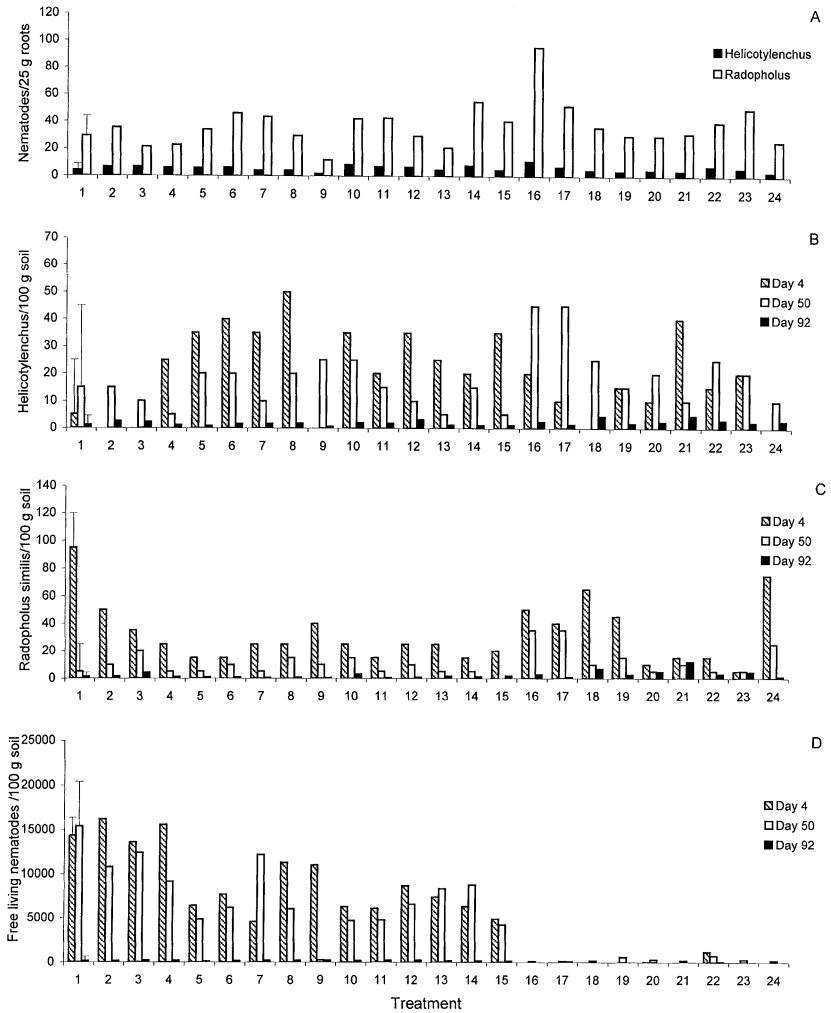

Figure 2. Populations of *Helicotylenchus multicinctus* and *Radopholus similis* in banana roots (A) and soil (B, C), and of free living nematodes in soil (D). Key to treatments in Table 1. Treatments # 1 to 15 contain wheat, # 16 is the untreated control, and # 17 to 24 do not contain wheat (see key to treatments 1 to 24 in Table 1). The total length of each bar (┬) represents least significant difference ($p \leq 0.05$) within a series.

the population of free-living nematodes had decreased dramatically (Figure 2D). Where only biocontrol microbes (for example the Bt + *Streptomyces* treatment # 21 as well as treatments # 17, 18, 19, 20, 22, 23) were applied, no equivalent buildup of free-living nematode populations occurred (Figure 2D). A rapid disappearance of the Bt was observed in the soil (Table 3).

Discussion

This study describes the effects of soil amendments, wheat mash with or without microbial nematode antagonists and nematicide, on growth of banana plants and populations of two plant-parasitic and free living nematodes. Wheat mash containing treatments increased plant growth and free living nematodes more than nematicide- and microbe-only treatments.

The effect of the wheat component on free living nematodes decreased significantly after 12 weeks for treatments without nematicide probably due to accelerated degradation of the organic amendment or build up of antagonistic fungi and bacteria. In the wheat plus nematicide treatments free living nematodes declined rapidly by the end of 4 weeks probably because the nematicide effectively decreased nematode survivability or longevity.

Table 3. Recovery of Bt strain CR371-rif^{+} from soil in banana experiment in Río Frío, Costa Rica

	Bt treatment concentration		No treatment
	1X	2X	
Day 4	$1.5 \times 10^5 \pm 1.3 \times 10^3$	$4.4 \times 10^5 \pm 1.7 \times 10^3$	0
Day 50	14 ± 6	53 ± 30	0
Day 92	0	0	0

Each datum (cfu/g soil $\pm$ SD) from 3 replicates.

The rapid decrease of the Bt strain suggests that effective biocontrol by the Bt (and perhaps the other microbes) may require more than one application. Support for this inference comes from greenhouse experiments in which 2 applications of the Bt spaced 30 days apart increased control of root-knot nematode (*Meloidogyne hapla*) on tomato (unpublished data). Rapid decline in population levels of the Bt and *Streptomyces* in soil has been observed (Esnard, 1995). This suggests that the wheat amendment (and not the microbes since their numbers declined rapidly) is most likely the more effective inducer of the process(es) that regulate free-living and plant-parasitic nematode populations (Figure 2D). Support for this also comes from the comparison of results for the wheat-only, wheat plus a microbe, and the corresponding microbe-only treatments (# 1, 2, 17 in Figure 2D).

The wheat mash and microbial cultures, directly or indirectly, through their decomposition products and activity, probably increased other organismal activity in the soil (Tomerlin, 1969) sufficiently to cause an increase in at least predatory organisms. Increased numbers of free-living nematodes were recorded and nematophagous fungi were observed in this study. Plant-parasitic nematode populations did not increase sufficiently in amended soil (Figures 2A, B) or their damage did not outweigh the plant growth promoting effect (Figure 1) from the probable higher nutrient source that became available in the form of wheat/microbial degradation by-products.

Observations on the wheat mash effect, low effect of the microbes added, rapid decline in the Bt strain, up to 1500- fold increase in free living nematodes, presence of nematophagous fungi, numbers of plant parasitic nematodes, and positive host growth response support the hypothesis of Duddington (1962), Linford (1937) and Linford et al. (1938) that organic amendments 'stimulated activity' antagonistic to nematodes. Wheat mash plus or minus microbe(s) increased overall numbers of bacteria in the soil which increased bacteriophagous nematodes that increased nematophagous fungi which in turn increased the incidence of infections in plant-parasitic nematodes. Overall host response was better probably because of the reduced numbers of root infections by plant-parasitic nematodes (Duddington, 1962).

It has been suggested that organic amendments release nematicidal substances directly or may control nematodes indirectly by toxic metabolites released by microorganisms associated with degradation of the material (Sikora, 1992). However, such was not the case in this study since free living nematodes increased dramatically in the wheat-treated soil (Figure 2D). Wheat alone (treatment # 1) or with bacterial/fungal amendments (treatments # 2 to # 15) most likely may have supplied a richer nutrient source to a broader spectrum of soil organisms. An increase in the numbers of these resident organisms in the soil could have regulated the plant-parasitic nematode population outside of the roots (Figures 1, 2, treatments 2 and 3 at least) and given the observed enhanced control, probably by the 'numerical response' mechanism described by other researchers (Jaffe and Muldoon, 1995; Soloman, 1949).

Sikora (1992) called for reevaluation of the mechanism of nematode control by organic amendments that lower plant parasitic nematode densities and stimulate egg-infecting soil antagonists. Treatment # 4 (viz. wheat + 2X *P. marquandii*) significantly increased the free living nematode population also ($p \geq 0.05$) (Figure 2D) and therefore cannot be working primarily by egg infection since *P. marquandii* is an egg parasite. Predacious fungal incidence increases when organic amendments are made to soil (Gray, 1988; Linford, 1937; Rodríguez-Kábana, 1991; Tomerlin, 1969). The *Paecilomyces* + *Bacillus* + *Streptomyces* treatment (# 23), however, effectively suppressed the burrowing nematode for 92 days (Figure 2C), but this suppression did not correlate with the best plant responses. Microbe-only amendments probably induce

host vigor and/or stimulate soil antagonistic activity to a lesser extent than certain organic amendments.

Gowen and Quénéhervé (1990) stated that because endoparasitic nematodes can complete their life cycles in banana corm and root tissue, the 'prospect of employing biological control agents seems remote'. However, it is necessary to take into account the fact that endoparasitic nematodes do spend a part of their life cycle in the soil, and it was probably during this phase that they were most vulnerable to the effects of the organic/microbial or nematicide treatments (Figure 2). The role of free living nematodes in the process(es) that promote plant health and in the mechanism of biological control of plant parasitic nematodes needs further investigation. Free living nematodes may be useful biological indicators of soil condition (Neher et al., 1995).

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