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Effect of Endophytic *Fusarium oxysporum* on Host Preference of *Radopholus similis* to Tissue Culture Banana Plants

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Abstract: The burrowing nematode *Radopholus similis* is one of the major constraints to banana (*Musa* spp.) production worldwide. Resource-poor farmers can potentially manage *R. similis* by using naturally occurring banana endophytes, such as nonpathogenic *Fusarium oxysporum*, that are inoculated into tissue culture banana plantlets. At present, it is unclear at what stage in the *R. similis* infection process the endophytes are most effective. In this study, the effect of three endophytic *F. oxysporum* isolates (V5w2, Eny1.31i and Eny7.11o) on *R. similis* host preference of either endophyte-treated or untreated banana plants was investigated. No differences were observed between the proportion of nematodes attracted to either root segments excised from endophyte-treated or untreated plants, or in experiments using endophyte-treated and untreated tissue culture banana plantlets. These results imply that the early processes of banana plant host recognition by *R. similis* are not affected by endophyte infection.

Key words: banana, choice test, endophyte, *Fusarium oxysporum*, host preference, *Musa*, *Radopholus similis*

The burrowing nematode *Radopholus similis* is among the most destructive pests of banana (Sarah et al., 1996). Plant yield losses as high as 50% have been observed under heavy nematode infestation (Speijer et al., 1999; Speijer and Kajumba, 2000). Currently, nematode management for resource-poor farmers mainly relies on the use of cultural methods such as pared and hot water-treated suckers (Speijer et al., 1995), tissue culture-derived planting material (Mateille et al., 1994; Sarah, 2000) and mulching (Talwana et al., 2003). None of these methods however offers complete nematode control. A combination of several complementary nematode management options would offer a more reliable nematode management strategy and has driven an increased impetus to develop alternative nematode management options that are sustainable and environmentally friendly.

A promising option currently under investigation for nematode management in banana is the use of antagonistic, endophytic micro-organisms (Sikora et al., 2003; Dubois et al., 2004; Gold and Dubois, 2005; Athman, 2006; Athman et al., 2006; Dubois et al., 2006). Endophytes are micro-organisms that spend part or all of their life cycle residing benignly inside host plant tissues (Wilson, 1995). Many endophytes form mutualistic relationships with their host plants, from which they obtain nutrients and in turn confer protection against biotic and abiotic stresses to the plant (Schulz and Boyle, 2005). In banana, naturally occurring endophytic *Fusarium oxysporum* isolates antagonized *R. similis* in vitro through the production of nematode-antagonistic metabolites (Dubois et al., 2004; Athman et al., 2006). Inoculation of some of these isolates into tissue culture plants resulted in improved plant growth and reduced nematode densities and damage (Dubois et al., 2004; Athman, 2006).

A crucial aspect of the potential for biological control of endophytic micro-organisms is to determine when and where during the nematode infection process they act, knowledge of which can lead to improvement of their efficacy. For example, endophyte-treated plants may produce substances that antagonize the action of the nematode's receptors during host searching, which may be used to decrease root invasion by nematodes (Perry, 1996).

Prior to infection and reproduction, nematodes need to migrate through the rhizosphere to the plant roots (Kaplan and Keen, 1980). Host recognition involves signals from the plant roots (Zuckerman and Jansson, 1984; Spiegel et al., 2001; Wuyts et al., 2006). The effect of endophytic *F. oxysporum* on the migration to and host recognition of banana plant roots by *R. similis* has not been investigated before. The objective of this study was to determine whether *R. similis* is differentially attracted to endophyte-treated and untreated tissue culture banana plants in order to understand at which point during the infection process the endophyte antagonizes the nematode. Three endophytic *F. oxysporum* isolates (V5w2, Eny1.31i and Eny7.11o) which have previously demonstrated nematode suppressing potential both in vitro and in vivo were selected for this study (Athman, 2006).

Materials and Methods

Experimental design: Two types of experiments were conducted to investigate host preference of *R. similis* towards endophyte-treated and untreated tissue culture banana plants. In a set of detached root experiments, root segments were excised from endophyte-treated and untreated banana plants and paired in a petri dish. In a set of intact plant experiments, endophyte-treated and untreated banana plants were paired in a polyvinyl chloride (PVC) tube. Each experiment was conducted

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twice. Both experiments comprised seven treatments that involved combinations of three endophyte-inoculated and untreated banana plants or roots in the following pairs: C-C, C-E1, C-E2, C-E3, E1-E1, E2-E2 and E3-E3, with E1 = V5w2, E2 = Eny1.31i, E3 = Eny7.11o and C = untreated plants or roots. Treatments were replicated four and five times for the detached root and intact plant experiments, respectively, in a completely randomized design.

Nematode cultures and banana plants: Pure *R. similis* cultures maintained on carrot disks were used as the source of nematode inoculum (Speijer and De Waele, 1997). Tissue culture banana plants of the East African highland banana cultivar Enyeru (genome group AAA-EA) were used in this study. The plants were micropropagated from sword suckers using a standard shoot-tip culture protocol for banana (Vuylsteke, 1998). When ready for weaning, plants were transferred to 250-ml plastic pots filled with 200 ml of nutrient solution to allow for enhanced root development prior to inoculation with the endophytes (Paparu et al., 2006). The nutrient solution was prepared by dissolving a water-soluble commercial fertilizer (Multifeed Classic, Gouws and Scheepers, Witfield, South Africa) at a rate of 2.5 g/liter in sterilized tap water. The plastic pots were transferred to a humidity chamber (1.92 x 1.59 x 1.23 m) in a screenhouse for 1 mon, during which the nutrient solution was renewed weekly.

Endophyte inoculation: Three *F. oxysporum* isolates (V5w2, Eny1.31i and Eny7.11o) were originally isolated from East African highland cooking banana plants in Uganda (Schuster et al., 1995) and were preserved in 25-ml tubes containing sterilized soil (Niere, 2001). The fungal isolates were grown on synthetic nutrient agar (SNA) medium (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar/liter distilled water) in 65-mm-diam. petri dishes in the laboratory (~25°C and a photoperiod of 12: 12 hr) for 1 wk. The SNA medium was supplemented with 10 mg chlortetracycline, 100 mg penicillin G and 50 mg streptomycin-sulphate/liter to prevent bacterial contamination. Half-strength potato dextrose broth (PDB) medium (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 12 g of PDB in 1 liter of distilled water. Aliquots of 100 ml of PDB were dispensed into 250-ml Erlenmeyer flasks and sterilized. After cooling, the flasks were inoculated with four to five 0.5-cm^3 disks of SNA medium containing fungal mycelium. Noninoculated PDB was used as a control treatment. Flasks were incubated in the laboratory for 7 d. Fungal spore suspensions were subsequently filtered through a 1-mm-aperture sieve to remove mycelial fragments and adjusted to provide a final spore count of 1.5 x 10^6 spores/ml under a haemocytometer. After 4 wk, plants were removed from the nutrient solution, and the roots trimmed to 10 cm in length. Roots were dipped in the different fungal spore suspensions or the control treatment for 2 hr. After fungal inoculation, plants were transplanted in steam-sterilized loamy soil into 250-ml plastic pots.

Detached root experiment: Because the root sections in the petri dishes were used for nematode extraction, they could not be used for determining absence or presence of *F. oxysporum* colonization. Prior to the experiment, absence (C treatment) or presence (E1, E2 and E3 treatments) of *F. oxysporum* colonization was confirmed using different sections from the same roots used in the experiments according to the procedure described by Paparu et al. (2006). Endophyte-inoculated and noninoculated plants were uprooted 1 mon after endophyte inoculation and rinsed free of soil. Healthy roots of the same age and size were selected from the plants and cut into 1-cm-long segments. Root segments from endophyte-inoculated and noninoculated plants were paired and placed on opposite sides in 90-mm-diam. petri dishes filled with a 50-mm-thick layer of moistened (3: 10 v/v sterile water: sand) sterile sand. The bottom of the petri dish was divided into two equal sections, A and B, and two root segments from each treatment pair were placed in each half. The distance between the root segments was 70 mm, and each root segment was placed 10 mm away from the wall of the petri dish. Petri dishes were closed and left to stand for 12 hr. Approximately 500 mixed *R. similis* stages (females, males and juveniles) in 0.3 ml water were placed in the center of the petri dish equidistant from the paired root segments. The sand and root segment in each of the sections of the petri dishes were separated 24 hr after nematode inoculation. Nematode extraction was separately carried out from the sand and root segments using a modified Baermann technique (Hooper et al., 2005). Extraction from the sand was carried out over 48 hr while nematodes were extracted from the roots for 24 hr after maceration in a Waring blender (Waring, Torrington, CT) for 15 sec. The nematode suspensions were concentrated in a 28-µm-aperture sieve, and the total numbers counted.

Intact plant experiment: Prior to the experiment, absence (C treatment) or presence (E1, E2 and E3 treatments) of *F. oxysporum* colonization was confirmed using selected roots from the same plants used in the experiments. The choice tube (22.5-cm-long, 5-cm-diam.) was a PVC tube divided into seven sections, each 2.5-cm long (Fig. 1; Prot, 1979). The sections were filled with moistened (3: 10 v/v sterile water: soil) steam-sterilized loamy soil and joined together with adhesive tape. Eight weeks after endophyte inoculation, plants of each treatment combination were paired at opposite ends of the choice tube. The bottom parts of the pots containing the plants were first removed to expose the roots, and the pots were fixed to distal sections (sections 1 and 7) of the choice tube. A 3-mm-diam. opening was drilled at the top surface of the center of each

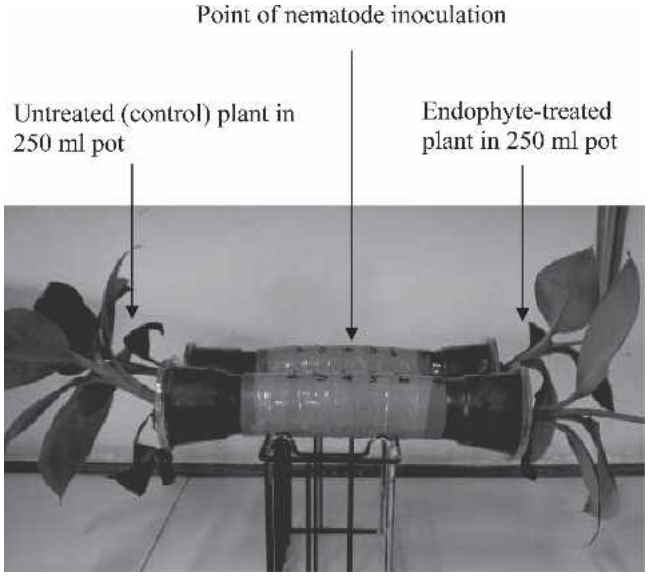

FIG. 1. Polyvinyl chloride (PVC) tube used for *Radopholus similis* host preference experiments involving intact endophyte (*Fusarium oxysporum*)-treated or untreated banana (*Musa* spp.) plants. The 17.5-cm-long tube comprised seven sections (each 2.5-cm long and 5 cm in diam.) joined end-to-end with adhesive tape and filled with steam-sterilized loamy soil. Endophyte-treated or untreated plants, in a 250-ml plastic pot with the bottom part removed, were paired and fixed in the first and last sections. Two thousand mixed (males, females and juveniles) stages of *R. similis* were added to the middle section equidistant to the two plants and extracted five days later.

PVC section to facilitate watering and nematode inoculation.

One week after transplanting the banana plants into the choice tube, soil in each of the sections was moistened with ~50 ml water, and 2,000 mixed stages of *R. similis* were pipetted into the soil in the middle section (section 4), equidistant to both plants. The tubes were maintained horizontally in the laboratory. The PVC sections were separated 5 d after introduction of the nematodes by cutting through the adhesive tape with a knife, and the number of nematodes in each section determined by extracting the nematodes from the soil over 48 hr using a modified Baermann funnel method (Hooper et al., 2005). The nematode suspensions were concentrated in a 28-µm aperture sieve prior to counting.

Data analysis: Nematode densities were tested for normality and homogeneity of variances prior to statistical analysis. For the detached root experiments, statistical analysis was performed on untransformed data, while for the intact plant experiments, nematode counts were transformed by square root (x + 0.5) prior to analysis. One-way analysis of variance (ANOVA) was used to demonstrate differences in the total number and proportions of nematodes recovered among treatment pairs. If different, treatment means were separated using Tukey's studentized range test. Within each treatment pair, differences in the number and proportions of nematodes between the root segments or intact plants were evaluated using paired t-tests (SAS Institute, Cary, NC).

RESULTS

Detached root experiment: The total number of nematodes recovered per petri dish was not different among treatment pairs (experiment 1: $P = 0.58$; experiment 2: $P = 0.58$) and ranged from 201 ± 10 to 260 ± 17 nematodes/petri dish and from 219 ± 25 to 287 ± 33 nematodes/petri dish in experiments 1 and 2, respectively (Table 1). This represented between 40.2 ± 2.1% to 52.1 ± 3.5% and 43.9 ± 4.9% to 57.4 ± 6.6% of the nematode inoculum added per petri dish in experiments 1 and 2, respectively.

Comparison between the proportions of *R. similis* that migrated towards opposite sections of the petri dish containing either an endophyte-inoculated or noninoculated root segment revealed no differences (Table 1). When two noninoculated root segments (C-C) were paired in opposite sides of a petri dish, the proportions of *R. similis* attracted to either root segment also did not differ. With one exception (E2-E2 in experiment 2), when two endophyte-inoculated root segments were paired (E1-E1, E2-E2 and E3-E3), no

TABLE 1. Total number and percentage of *Radopholus similis* attracted to banana (*Musa* spp.) root segments excised from either an endophyte (*Fusarium oxysporum*)-treated or untreated plant paired in a petri dish.

Treatment pair[a]	Experiment 1				Experiment 2			
	Total *R. similis*[b]	Petri dish section		*P*	Total *R. similis*[b]	Petri dish section		*P*
		A (%)	B (%)			A (%)	B (%)	
C-C	234 ± 13	57.1 ± 3.3	42.8 ± 3.4	0.11	238 ± 25	54.5 ± 4.4	45.5 ± 4.4	0.35
C-E1	239 ± 8	52.2 ± 5.7	47.8 ± 5.7	0.69	251 ± 11	53.4 ± 5.8	46.6 ± 5.8	0.56
C-E2	201 ± 10	50.7 ± 1.8	49.3 ± 1.8	0.75	220 ± 25	48.8 ± 5.4	51.1 ± 5.4	0.28
C-E3	231 ± 30	53.8 ± 4.7	46.2 ± 4.7	0.42	256 ± 19	61.4 ± 3.6	38.5 ± 3.5	0.30
E1-E1	223 ± 21	56.3 ± 2.4	43.7 ± 2.4	0.13	237 ± 29	56.2 ± 5.8	43.7 ± 5.8	0.33
E2-E2	238 ± 39	43.4 ± 3.3	56.6 ± 3.2	0.13	287 ± 33	36.5 ± 4.7	63.5 ± 4.7	0.041
E3-E3	260 ± 17	47.3 ± 6.3	52.7 ± 6.3	0.70	267 ± 18	41.6 ± 3.1	58.3 ± 3.1	0.059

Nematodes were extracted from the sand and root setments in opposite sections, labelled A and B, of the petri dishes. The number of nematodes in each section was expressed as a percentage of the total nematodes recovered per petri dish.

[a] C = untreated plant, E1 = isolate V5w2, E2 = isolate Eny1.31i, E3 = isolate Eny7.11o; the first and second treatments of each treatment combination correspond with sections A and B, respectively.

[b] Total nematodes extracted from the sand and root in both sections A and B, n = 4.

differences were observed in the number of *R. similis* that migrated towards either root segment.

Intact plant experiment: The proportion of *R. similis* that migrated from the point of inoculation in the choice tubes did not differ among any of the treatment pairs for both experiments ($P \geq 0.15$; Table 2). In experiment 1, the proportion of nematodes that migrated from the point of nematode inoculation ranged from 30.5 ± 2.7% in treatment pair C-E3 to 43.9 ± 5.6% in treatment pair E3-E3. In experiment 2, the proportion of nematodes that migrated from the point of inoculation ranged from 40.0 ± 1.6% in treatment pair E3-E3 to 57.6 ± 10.1% in treatment pair C-E3.

Between each of the treatment pairs, the proportion of *R. similis* that migrated to opposite sides of the inoculation point did not differ in both experiments, except for the treatment pair E3-E3 (Table 2).

In both experiments, the total number of nematodes was significantly different among the various PVC sections ($P < 0.0001$; Fig. 2). Most nematodes were found in the middle section (section 4), the point of nematode inoculation, with progressively fewer nematodes in sections further from the point of nematode inoculation. The numbers of nematodes in PVC sections at the same distance from the point of nematode inoculation (sections 3 and 5, sections 2 and 6 and sections 1 and 7) did not differ within any of the treatment pairs ($P \geq 0.05$).

The total number of nematodes recovered in the whole choice tube (including those that migrated and those that did not migrate) differed among the treatments in experiment 1 ($P = 0.0008$) but not in experiment 2 ($P = 0.67$; Table 3). In experiment 1, a higher total number of *R. similis* was found in the C-C treatment pair than in the E1-E1 and E3-E3 treatment pairs.

Discussion

Using our experimental conditions, *F. oxysporum* endophyte-treated roots of tissue culture banana plants did not influence host preferences by *R. similis*. This is evident from the lack of repulsion or attraction of *R. similis* in experiments conducted with detached root segments and intact banana plants. *Radopholus similis* showed no preference when facing a choice between endophyte-treated and untreated plants or roots, in that a similar proportion migrated towards either plant type.

This suggests that the biological control potential for some endophytes antagonistic to nematodes in banana, such as *F. oxysporum*, is post-infectional. In other studies, biological control agents other than endophytes also did not affect host preference, and their nematode-antagonizing effect was post-infectional. In sugar beet (*Beta vulgaris*), Oostendorp and Sikora (1989, 1990) reported a reduction in egg hatch and early root infection by the sugar beet nematode *Heterodera schachtii* after seed treatment with antagonistic rhizobacteria. However, treatment of sugar beet seedlings with the rhizobacteria did not alter migration of *H. schachtii* J2. Contrasting results were obtained by Bernard and Gwinn (1991), who reported that more *Pratylenchus scribneri* migrated towards tall fescue (*Festuca arundinacea*) free of the endophyte *Neotyphodium coenophialum* when both endophyte-infected and endophyte-free root segments were paired in a petri dish. Interestingly, in a study by Edmund and Mai (1967), pathogenic *F. oxysporum* influenced movement of plant-parasitic nematodes. They demonstrated that alfafa (*Medicago sativa*) seedlings infected by pathogenic *F. oxysporum* attracted more *Pratylenchus penetrans* than healthy plants. Probably, plants react differently to pathogenic infections, increasing their susceptibility to secondary infection.

From the current study, treatment of banana plants with *F. oxysporum* does not seem to interfere with the nematode's ability to locate and migrate towards plant roots. The fact that *R. similis* was equally attracted to endophyte-treated and untreated plants may imply that endophyte infection may not have influenced root exu-

Table 2. Percentage of *Radopholus similis* that migrated towards either intact endophyte (*Fusarium oxysporum*)-treated or untreated banana (*Musa* spp.) plants paired in a polyvinyl chloride (PVC) tube.

	Experiment 1			Experiment 2		
	PVC section			PVC section		
Treatment pair[a]	A	B	*P*	A	B	*P*
C-C	21.9 ± 1.4	17.4 ± 1.2	0.10	23.5 ± 3.5	25.8 ± 3.2	0.59
C-E1	22.4 ± 4.4	16.4 ± 3.5	0.49	18.9 ± 3.5	27.8 ± 3.2	0.23
C-E2	17.9 ± 3.2	20.7 ± 1.7	0.35	17.5 ± 2.3	24.1 ± 3.3	0.16
C-E3	16.2 ± 3.8	14.4 ± 3.5	0.80	30.7 ± 10.5	26.9 ± 2.4	0.75
E1-E1	21.7 ± 3.7	22.4 ± 4.1	0.93	31.7 ± 10.4	17.5 ± 5.3	0.43
E2-E2	12.1 ± 1.5	27.9 ± 4.9	0.067	21.6 ± 5.4	24.4 ± 3.5	0.72
E3-E3	31.0 ± 4.7	11.9 ± 1.6	0.011	25.5 ± 2.6	14.9 ± 1.3	0.049

Endophyte-treated and untreated plants were placed on opposite sides of a choice tube divided into seven equal sections. Section A and section B refer to the three left and three right sections, respectively.

[a] C = untreated plant, E1 = isolate V5w2, E2 = isolate Eny1.31i, E3 = isolate Eny7.11o; the first and second treatments of each treatment combination correspond with sections A and B, respectively.

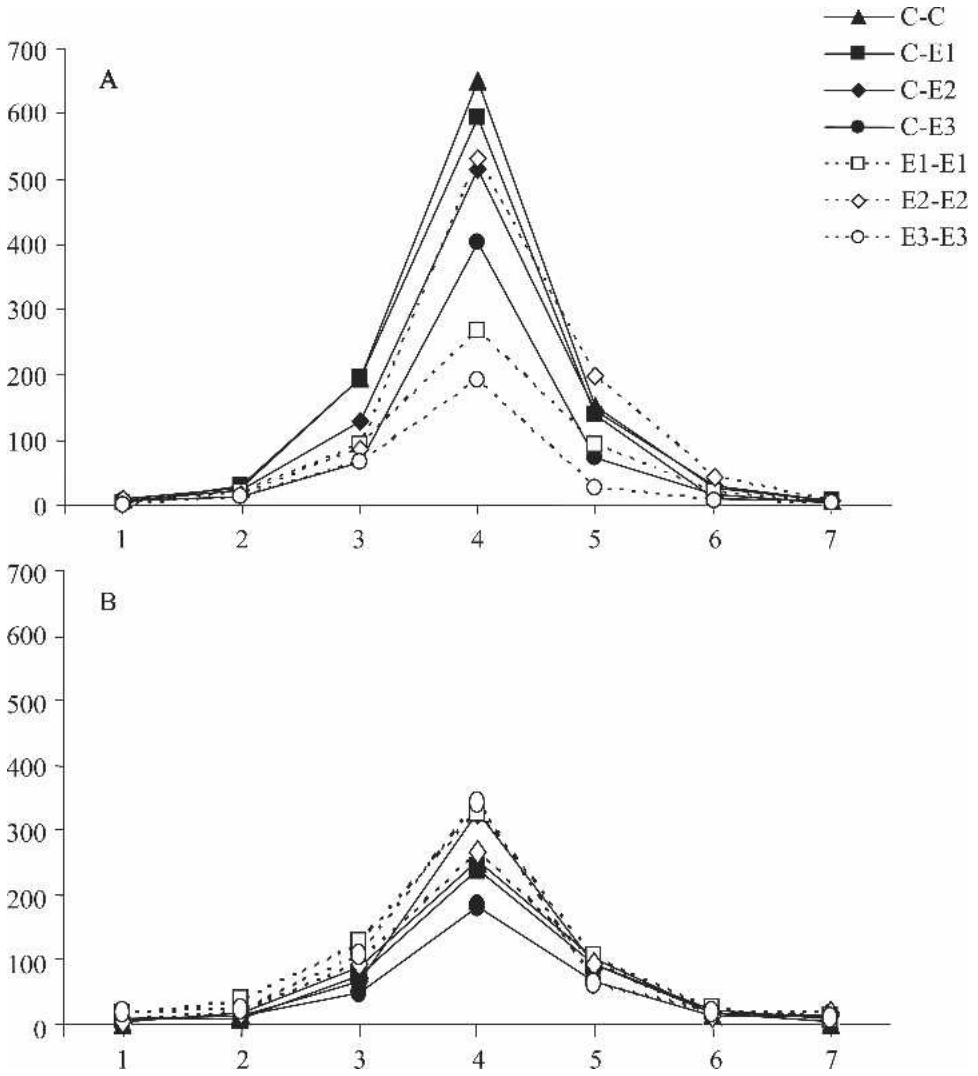

FIG. 2. Migration of *Radopholus similis* towards endophyte (*Fusarium oxysporum*)-treated and untreated banana (*Musa* spp.) plants paired in a polyvinyl chloride (PVC) tube apparatus. The choice tube was divided into seven sections (2.5-cm-long, 5-cm-diam.) and 2,000 mixed (males, females and juveniles) stages of *R. similis* were introduced in the middle section of the choice tube (section 4) at equal distances from both plants placed in sections 1 and 7. C = control, E1 = isolate V5w2, E2 = isolate Eny1.31i, E3 = isolate *Eny7.11o*. A and B represent experiments 1 and 2, respectively.

dation by the plants. Plants are known to produce root exudates that may act as attractants or repellents of nematodes, while nematodes possess sensory organs that act as receptors allowing them to locate their hosts at a distance (Prot, 1980). However, endophytes inoculated in tissue culture plants may result in *R. similis* mortality during migration towards the plant, evidenced by the low total number of nematodes recovered in treatments involving two endophyte-treated plants in at least one of the intact plant experiments.

This is the first study that has attempted to elucidate some of the nematode infection stages at which endophytic *F. oxysporum* are effective against *R. similis*. We have shown that endophyte inoculation of banana plants does not influence host preference by *R. similis*. Thus, since the initial stage of the nematode infection process is not altered, we hypothesize that the reduction of *R. similis* observed by Sikora et al. (2003), Du-

TABLE 3. Total number of *Radopholus similis* recovered per treatment pair in two intact banana (*Musa* spp.) plant experiments using a polyvinyl chloride (PVC) tube. Treatments pairs comprised untreated plants and plants inoculated with endophytic *Fusarium oxysporum*.

Treatment pair[a]	Experiment 1		Experiment 2	
C-C	1,071.0 ± 104.8	a	506.0 ± 57.9	a
C-E1	977.4 ± 133.9	ab	480.6 ± 72.6	a
C-E2	855.2 ± 47.7	ab	553.4 ± 142.8	a
C-E3	577.5 ± 169.2	abc	362.8 ± 145.6	a
E1-E1	501.4 ± 136.7	bc	640.8 ± 105.7	a
E2-E2	889.2 ± 83.7	ab	525.8 ± 108.8	a
E3-E3	309.4 ± 128.2	bc	586.4 ± 85.9	a
P	0.0008		0.67	

[a] C = untreated plant, E1 = isolate V5w2, E2 = isolate Eny1.31i, E3 = isolate Eny7.11o.

[b] Total nematodes recovered per treatment pair (those that migrated plus those that did not migrate).

Values represent mean ± SE, n = 5. In each experiment, means followed by the same letter are not different at $P \geq 0.05$ (Tukey's studentized range test).

bois et al. (2004), Gold and Dubois (2005), Athman (2006) and Dubois et al. (2006) in plants treated with endophytic *F. oxysporum* occurs at a later, post-infectional stage.

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