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Aphelenchus avenae and Antagonistic Fungi as Biological Control Agents of *Pythium* spp.

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To examine the control effect of damping-off on radish caused by *Pythium* spp., researchers used the isolates of a fungivorous nematode, *Aphelenchus avenae*, and antagonistic fungi, *Trichoderma* spp. These were used as biocontrol agents, either alone, or in combination. Growth rates of the *A. avenae* isolates and fungal damages by the nematodes varied depending on *Trichoderma* spp., which contained lower *T. koningii* and *T. virens* cultures than other *Trichoderma* cultures. *Pythium* spp. were damaged by all five *Aphelenchus* isolates, but the multiplication rate of nematode isolate Aa-3 was very poor. Antibiotic activity of *T. virens* and *T. harzianum* to *Pythium* spp. was stronger than that of *T. koningii*. Control efficacy against damping-off of radish was most enhanced under the treatment using the nematode-*T. harzianum* combination. On the contrary, the combinations of the nematodes and *T. virens* or *T. koningii* mostly did not increase or decreased their control effect vis-à-vis that of the nematodes or antagonistic fungi being used alone. The results suggest that the fungivorous nematodes may play a leading role in the disease control, and that the activity of the fungivorous nematodes may be activated by *T. harzianum*, but inhibited by *T. koningii* and *T. virens*.

Keywords : *Aphelenchus avenae*, biocontrol, damping-off of radish, *Pythium* spp., *Trichoderma* spp.

In agroecosystem, soil nematodes participate in many interactions affecting crop plants (Freckman and Casewell, 1985). Fungivorous nematodes feed on many different species of fungi including saprophytic, pathogenic, beneficial, and mycorrhizal fungi growing in the rhizosphere. Among fungivorous nematodes such as the *Aphelenchus avenae*, *Aphelenchoides* spp., *Tylenchus* spp., and *Ditylenchus* spp., only *A. avenae* has been considered and used as a biological control agent against pathogenic fungi (Barnes et al., 1981; Freckman and Casewell, 1985; Rhodes and Linford, 1959).

Other fungal feeding nematodes are facultative plant parasites. Other microorganisms such as bacteria and actinomycetes are poor food sources for *A. avenae* (Walker, 1984). *A. avenae* was also found in a diseased portion; however, it was suggested that the nematode was not associated with the disease, but with the microorganisms already existing (Rhodes and Linford, 1959).

Trichoderma spp., including *Gliocladium virens* which was recently named as *Trichoderma virens*, are well-known antagonistic fungi useful in controlling soil-borne diseases. They have been used against the disease caused by *Pythium* spp. (Howell, 1991; Howell and Stipanovic, 1983; Hwang et al., 1996; Park et al., 1995; Sivan et al., 1984; Wolffhechel and Jensen, 1991). They have also been used against the disease caused by *Rhizoctonia solani* (Elad et al., 1981; Mihuta-Grimm and Rowe, 1986) and by both *Pythium* spp. and *R. solani* (Cliquet et al., 1996; Howell, 1982; Kim, 1994; Lumsden and Locke, 1989).

The principal control mechanisms of the biocontrol agents are mycoparasitism and antibiosis (Howell, 2003). Especially the antibiotic mechanism has been evidently related to the biological control. For example, mutants with increased or decreased antibiotic production show a corresponding effect on biocontrol (Howell and Spinovic, 1983, 1995; Howell et al., 1993). Mutants of *T. virens* that do not produce gliotoxin are reduced in their ability to control *Pythium* damping-off (Wilhite et al., 1994).

Disease suppression by biocontrol agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environments (Handelsman and Stabb, 1996). The control efficacies of biocontrol agents against plant diseases fluctuate greatly depending upon crops, fields, environmental conditions, and species and strains of target organisms. Different biocontrol agents can be mixed and applied to give better control efficacy against plant pathogens. Both should be compatible and should not give a considerable harmful effect to each other.

Materials and Methods

In this study, biocontrol effect on *Pythium* damping-off was examined by using the fungivorous nematode, *A. avenae*, and the

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antagonistic fungi, *Trichoderma* spp., alone or in combination. Growth of the nematode population on the antagonistic fungi was compared with one another and that on the plant pathogenic fungi to examine selective activity of the nematode against the pathogens. The control efficacy of both biocontrol agents was explained based on their fungivorous or antagonistic activity to the pathogens and to the other biocontrol organism.

Biocontrol organisms and pathogens. Five isolates of *A. avenae* were used in this experiment. *A. avenae* isolate 1 (Aa-1) was isolated from a tobacco field in Changsung, Chonbuk province. Isolates 2 (As-2), 3 (Aa-3) and 4 (Aa-4) were taken from the weedy fields in Suwon, and isolate 5 (Aa-5), from a potato field in Daekwallyoung, Korea. Nematodes were extracted by the Baermann funnel and transferred to potato-dextrose agar (PDA) for multiplication on which *Fusarium* sp. was growing. Nematodes cultured on the medium were extracted by the Baermann funnel and collected on a 500-mesh sieve to remove fungal spores.

Six isolates of *Trichoderma* spp., namely: *T. aureoviride* (Taur), *T. harmatum* (Tham), *T. harzianum* (Thaz), *T. koningii* (Tkon), and *T. pseudokoningii* (Tpsk), and *Trichoderma* (*Gliocladium*) *virens* (Tvir) were used in this experiment as antagonistic fungi. These were provided by the Plant Disease Epidemiology Laboratory at the Seoul National University. Two *Pythium* isolates, *P. aphanidermatium* (Paph) and *P. ultimum* (Pult) were provided by Dr. Jin Won Kim at the Department of Environmental Horticulture, The University of Seoul, Korea.

Culture of *A. avenae* on antagonistic and pathogenic fungi. Each *A. avenae* isolate was suspended in sterilized water and transferred into the 6-day-old and 13-day-old antagonistic fungal cultures of *Trichoderma* spp. and into the 6-day-old cultures of *Pythium* spp. Fifty nematodes were inoculated on each fungal culture and incubated at room temperature. Fifteen days after inoculation, conditions of fungal colonies were examined visually. Nematodes were extracted by the Baermann funnel and collected on 500-mesh sieve to remove fungal spores. Nematodes were counted under a stereomicroscope. Three replications were used for each fungal culture.

Antibiotic effect of antagonistic fungi. Three antagonistic fungi: Thaz, Tkon, and Tvir were selected to test their antibiotic effect against the pathogens. Because Tkon and Tvir were less damaged by *A. avenae* than Thaz, the combined treatment of these antagonistic fungi with the fungal feeding nematode would differentiate the control efficacies of the two (fungivorous nematodes or antagonistic fungi).

The bottom of aluminum ring (3 cm in diameter, 0.8 cm in height) was sealed with aluminum foil, and sterilized at 121°C for 20 min. Warm PDA was poured into the aluminum ring and hardened at room temperature. Each antagonistic fungus was inoculated on the medium, and incubated at 25°C. Two days after inoculation, when the antagonistic fungus grew enough to be seen, mycelial discs (5 mm in diameter) of Paph and Pult were inoculated on the other side of the medium and incubated at 25°C. Three days after inoculation, the growth of the pathogens was examined visually. Three replications were used for each antagonistic fungus - pathogen combination.

Effects of *A. avenae* and antagonistic fungi. Radish, *Raphanus sativus* L. cv. Taesanmu (Jeil Seed & Agricultural Products Co., Korea), was used as test plant. Oatmeal-sand medium (oatmeal 1: sand 20: distilled water 4) was used in preparing the pathogen inocula of Paph and Pult. The medium was autoclaved for 20 minutes at 121°C. Mycelial plugs of *Pythium* spp. grown on potato carrot agar (PCA) at 25°C for 2 days were inoculated into the oatmeal-sand medium. The inoculated medium was incubated at 25°C for 14 days. The culture medium was ground in a blender and used as the fungal pathogen inoculum.

The inoculum of the antagonists was made up of rice hull added with tap water in the ratio of 1:1.5 (v/v). The moistened rice hull was autoclaved for one hour at 121°C two times. The medium was inoculated with Thaz, Tkon, and Tvir, and incubated for 6 days at 23°C.

River sand soil was autoclaved for 1 hour at 121°C two times. The soil was mixed with the fungal culture medium of Pult and Paph in the ratio of 5:3 (v/v) by which about 50% damping-offs had occurred in a preliminary test. The antagonistic fungi (Thaz, Tkon, and Tvir), which were grown for 6 days on the rice hull medium, were applied into the soil (5 g/L and 1 g/L of soil for the first and second experiments, respectively), and mixed thoroughly.

Radish seeds were planted in the soil in 11 × 9 × 4.5 cm plastic containers. The nematode suspension (5,000 nematodes per container) was then poured into the soil and grown at room temperature. Three replications with 10 seeds in each container were used for each treatment. Ten days later, incidence of damping-off was examined.

Data analysis. Data from repeated experiments were tested by one-way variance analysis followed by the least significance difference (LSD) test.

Results

Damage on the fungi by *A. avenae* feeding. All of the antagonistic and pathogenic fungi used in this study grew well on PDA. Damage on fungal colonies by fungivorous nematodes was initially noted by the breaking down of the aerial mycelium and forming of sunken areas in the colonies. Later, when the fungal colonies were completely damaged, they had watery surface without aerial mycelium.

In *Pythium* spp., all of the fungal colonies were damaged greatly by the nematode infestation without remarkable variations (Table 1). All *Pythium* spp. tested (Paph and Pult) were damaged almost completely by Aa-2, Aa-4, and Aa-5. However, there were less severe damages in Paph by Aa-1 and Aa-3. In the antagonistic fungi, there were significant differences in damage by *A. avenae* among the fungal isolates. In Thaz, Tham, Taur, and Tpsk, the fungal colonies were damaged greatly by the nematodes (Table 1). However, in Tkon and Tvir, the fungal colonies were damaged a little by the nematode infestation; they showed almost intact colonies.

Growth of *A. avenae* on fungal cultures. Multiplication of

Table 1. Damage of antagonistic (*Trichoderma* spp.) and pathogenic (*Pythium* spp.) fungi by *Aphelenchus avenae* isolates

A. avenae	Mycelial damage[a]							
	Antagonistic fungi						*Pythium* spp.	
	Taur[b]	Tham	Thaz	Tkon	Tpsk	Tvir	Paph	Pult
Aa-1[c]	+++	+++	+++	±	++	±	++	+++
Aa-2	+++	+++	+++	±	+++	±	+++	+++
Aa-3	+++	++	++	±	+++	±	++	+++
Aa-4	++	++	++	+	+	±	+++	+++
Aa-5	++	+++	++	+	+++	±	+++	+++

[a] +++: Mycelium damaged completely, ++: more than 50%, +: less than 50%, ±: little damaged. In antagonistic fungi, nematodes were inoculated 6 and 13 days after fungus culturing (50 nematodes were inoculated per culture).
[b] Taur: *T. aureoviride*, Tham: *T. hamatum*, Thaz: *T. harzianum*, Tkon: *T. koningii*, Tpsk: T. *pseudokoningii*, Tvir: *Trichoderma virens*, Paph: *P. aphanidermatum*, Pult: *P. ultimum*.
[c] Aa-1-5: *A. avenae* isolates 1-5.

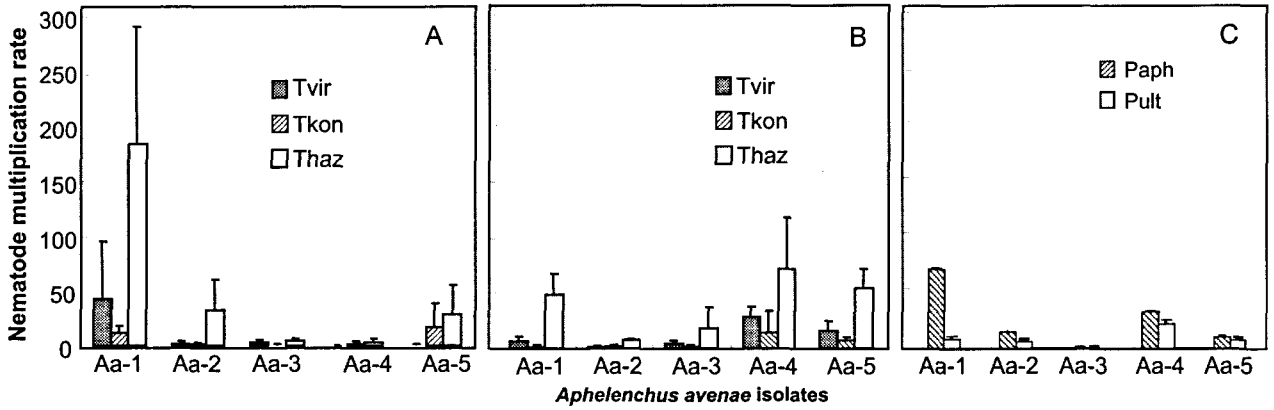

Fig. 1. Multiplication of *Aphelenchus avenae* isolates on the cultures of antagonistic fungi (inoculated on 6-day-old fungal cultures (**A**) and 13-day-old fungal cultures (**B**)) and on cultures of *Pythium* spp. (Paph: *P. aphanidermatum*, Pult: *P. ultimum*) (**C**). Aa-1-5: *A. avenae* isolates 1-5. Thaz: *T. harzianum*, Tkon: *T. koningii*, Tvir: *T. virens*.

A. avenae on Thaz, Tkon, and Tvir varied greatly, depending on the fungal isolates and also among the nematode isolates. Aa-1 was the fastest growing nematode among all isolates tested (Fig. 1). The nematode growth rates were higher in Thaz than in those of the Tkon and Tvir on which little mycelial damage caused by the nematodes occurred. The growth rate of Aa-1 was highest in the culture of Thaz (inoculated on 6-day-old culture). Its multiplication rate was 182 folds, but the population of Aa-3 on Tkon (inoculated on 13-day-old culture) was 0.4 times of the initial one, indicating that the nematode population was decreased in the antagonistic fungal culture.

Four isolates of *A. avenae* (Aa-1, 2, 4 and 5) had population growths on all of the *Pythium* cultures used in this experiment (Fig. 1C). However, Aa-3 showed poor growths on the two *Pythium* cultures. Multiplication rates of Aa-1 and Aa-4 were high on Paph, these being increased to more than or around 50 times. In the other nematode-fungus combinations, population increase in the cultures ranged from lowest 2.1 times to highest 24.4 times.

Antibiotic effect of antagonistic fungi. Mycelial growth of *Pythium* spp. (Paph and Pult) was inhibited by all of the antagonistic fungi (Thaz, Tkon, and Tvir), while mycelia grew fully in the control media (Fig. 2). Thaz and Tvir showed stronger antibiotic activity to *Pythium* species than Tkon on which both Paph and Pult formed some mycelia.

Effects of *A. avenae* and antagonistic fungi. In this study, all the nematode isolates tested *in vitro*, except Aa-4, were applied to control the damping-off disease caused by *Pythium* spp., because Aa-4 and Aa-5 seemed to have almost identical cultural characteristics with *Trichoderma* and *Pythium* cultures. Besides, the number of Aa-4 inoculum was not enough for the control experiments. Antagonistic fungi, Thaz, Tkon, and Tvir were selected because they showed different antagonistic effects on *Pythium* spp. and differed as food for the nematode growth. In the second experiment, nematode isolates Aa-1 and Aa-2 were tested again as a replication of the first experiment.

Damping-off was reduced by either the nematodes or the antagonistic fungi. Control effects of the fungivorous

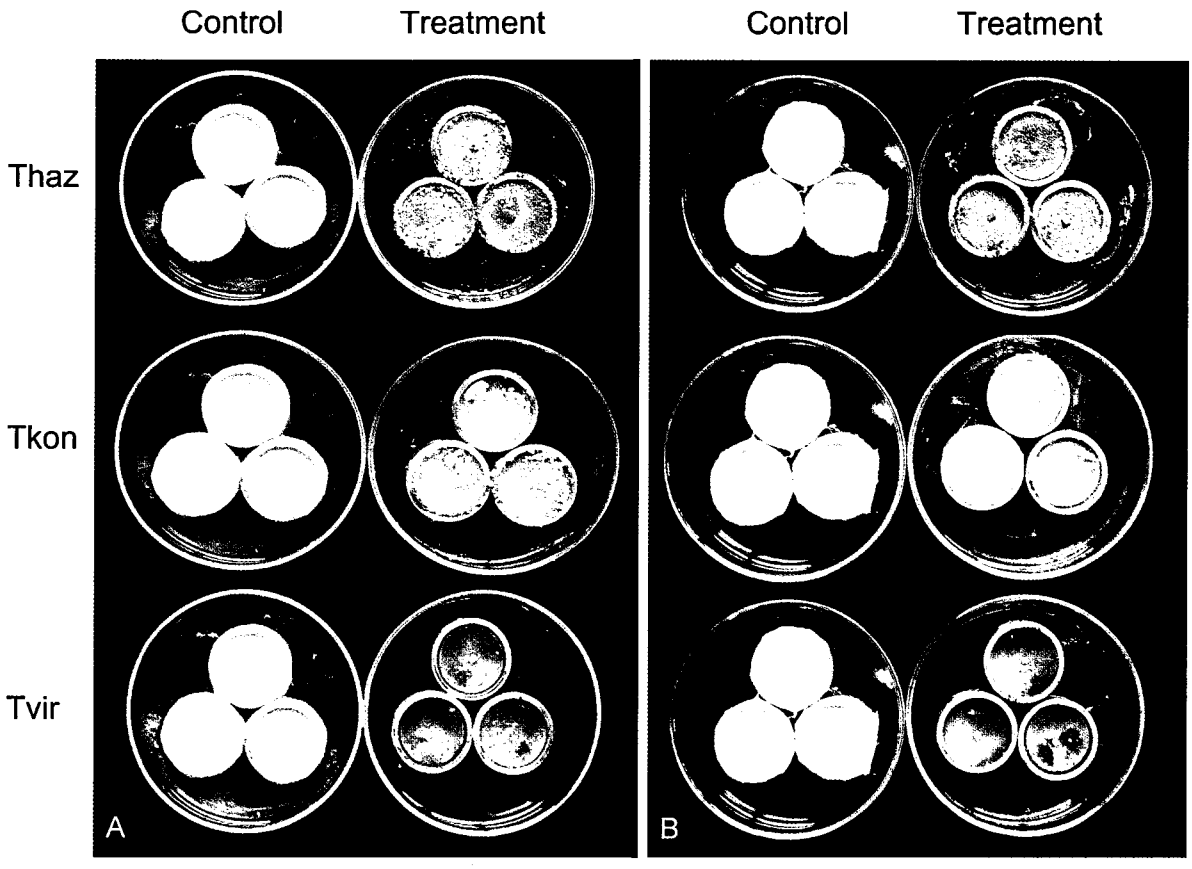

Fig. 2. Antibiotic effect of *Trichoderma* spp. on *Pythium aphanidermatium* (**A**) and *P. ultimum* (**B**). Thaz: *T. harzianum*, Tkon: *T. koningii*, Tvir: *T. virens*. Note full mycelial growth of *Pythium* spp. on the control media, but no or minimum mycelial growth on Thaz and Tvir. A little mycelial growth was observed on Tkon.

nematodes alone were similar among the nematode isolates, ranging from 32% to 45%, and those of antagonistic fungi alone, from 21% to 32% (Table 2). Dual treatments of both nematodes and antagonistic fungi increased or reduced the control effects, compared with either the nematode or the fungus alone. Treatment of Thaz together with nematode isolates generally increased the control effect by 5-34%, except that of Aa-5, while the addition of Tvir and Tkon generally did not significantly enhance the effect or reduced the biocontrol efficacy.

In the second experiment, it is confirmed that the amendment of Thaz with the nematodes significantly enhanced the control efficacy against damping-off (Table 2). The combination of Tvir and Tkon with the nematodes also showed control effects similar to those of the first experiment; it either showed the same or decreased control efficacy relative to the nematode alone.

Discussion

Five isolates of *A. avenae* were used in this experiment, each of which was obtained from different fields. Morphological characters of the nematode isolates were similar to one another, and identical to *A. avenae* based on shapes of body, tail, and head, and various measurements (unpublished data). The five isolates of *A. avenae* grew well on the antagonistic fungi as well as on the pathogenic fungi with some exceptions. The nematode population growth was more variable on the antagonistic fungi than on the pathogenic fungi. The growth rate of *A. avenae* was low on the cultures of Tkon and Tvir, and accordingly, the antagonistic fungi Tkon and Tvir were little damaged by *A. avenae* compared with other *Trichoderma* spp. (Table 1).

Compared with mycelial damages, the nematode multiplication rates were always higher in Thaz than those in *Pythium* spp. (Fig. 1), suggesting that the antagonistic fungus might be still supporting the nematodes with living hyphal cells, for the nematode to exert full feeding activities. This might be that the more effective control combination would be that of the fungivorous nematodes and Thaz, than when nematodes or antagonistic fungus were used alone.

As *Trichoderma* species have long been focused as most potent biocontrol agents for fungal diseases, other biotic

Table 2. Control of damping-off of radish caused by *Pythium aphanidermatium* (Paph) and *P. ultimum* (Pult) with *Aphelenchus avenae* isolates and biocontrol fungi

Treatments		Control efficacy (%)			
Aphelenchus avenae isolate[a]	Biocontrol fungus[b]	Experiment 1		Experiment 2	
		Paph[c]	Pult	Paph	Pult
Aa-1	Thaz	72 Y	50 WX	60 WX	75 YZ
	Tko n	25 ST	39 UV[d]	41 U	49 X
	Tvir	47 VW	19 S	50 V	30 UV
	No fungus	57 X	45 VW	42 U	43 V
Aa-2	Thaz	43 V	58 XY	65 XY	81 Z
	Tkon	6 Q	34 UT	50 V	19 T
	Tvir	55 WX	40 UV	67 Y	38 VW
	No fungus	34 U	44 VW	55 VW	34 UVW
Aa-3	Thaz	81 Z	68 Z	–[e]	–
	Tkon	15 R	60 YZ	–	–
	Tvir	0 Q	6 R	–	–
	No fungus	23 RST	34 TU	–	–
Aa-5	Thaz	30 TU	21 S	–	–
	Tkon	2 Q	3 R	–	–
	Tvir	28 STU	5 R	–	–
	No fungus	21 RS	32 TU	–	–
Control	Thaz	32 U	30 T	55 VW	67 Y
	Tkon	25 ST	21 S	72 Z	25 TU
	Tvir	28 STU	32 TU	55 VW	30 UV
	No fungus	0 Q	0 R	0 T	0 S
	LSD	8.4	8.7	6.7	9.3

[a] Aa-1-5: *A. avenae* isolates 1-5 (five thousand nematodes were applied to rhizosphere soil for each container).
[b] Thaz: *T. harzianum*, Tkon: *T. koningii*, Tvir: *Trichoderma virens*
[c] Paph: *P. aphanidermatum*, Pult: *P. ultimum*.
[d] Means (of three replications) followed by the same letters in a column are not significantly different at P = 0.05 according to least significance difference (LSD) test.
[e] Not tested

factors such as bacteria have generally been considered as affecters to their control activities (Bin et al., 1991; Dandurand and Knudsen, 1993; Hubard et al., 1983). Bae and Knudson (2001) reported that sclerotia of *Sclerotinia sclerotiorum* were less colonized by *T. harzianum* when a fungus-feeding nematode, *Aphelenchoides* sp., was added. If this were true in the *Aphelenchus-Trichoderma* combinations in our study, *Aphelenchus*-Tkon or *Aphelenchus*-Tvir would be the more effective combinations in controlling damping-off than *Aphelenchus*-Thaz, because the nematodes damaged Thaz more than Tkon and Tvir. However, the results were contrary to this hypothesis. In the first experiment, we used 5 g of *Trichoderma* inoculum for one liter of soil, and observed some phytotoxicity to radish seedlings. One possible explanation is that the fungivorus nematodes may have reduced this phytotoxicity by feeding and damaging Thaz more. However, in the second experiment, 1 g of *Trichoderma* inoculum was used and no phytotoxicity by the inoculum was observed. The increased control efficacy of the combined treatment cannot be explained by mutual antagonisms. Multiplication rates were high on Thaz (Fig. 1), which increased feeding activity of *A. avenae* in the combined treatment to give better control effect.

The aluminum ring test in our study showed that antibiotic materials may be involved in inhibiting the pathogen growth. Tkon had lesser inhibitory effect on *Pythium* spp. than Thaz and Tvir. Poor nematode multiplications of Tkon and Tvir may be due to either the low feeding preference by the nematodes to the fungi or the toxic effect exerted by the antagonistic fungi. *Trichoderma* spp. reduce egg production (Windham et al., 1989), possess nematicidal or nematostatic components (Khan and Saxena, 1997), and inhibit nematode mobilization and egg hatching (Sharon et al., 2001) in root-knot nematodes. This indicates a strong possibility that *A. avenae* may be adversely affected by *Trichoderma* spp., especially by Tkon and Tvir.

The generally reduced control efficacy of the combined treatments of fungivorous nematodes with Tkon or Tvir in our study may be derived from the inhibited nematode activity mentioned above. Thaz, enhanced the control efficacy of the nematodes, which may be derived from adding its antifungal activity (stronger than Tkon) against *Pythium* with little adverse influence on the nematodes. Control efficacy for the disease was not changed much or a little decreased in the nematodes-Tvir combination. This is probably because the antagonistic fungus was strongly antifungal to *Pythium* spp. compared to Tkon, although the fungivorous nematodes did not feed well on the antagonistic fungus like Tkon.

The fact that in the biocontrol, the activity of the fungivorous nematodes may be more influenced by fungal antagonists than that of the fungal antagonists by the nematodes suggests that the nematodes may play a leading role in the biological relations with the fungal pathogens. However, this may be changed in different soil environments because the place for root disease development is the interface of root and soil known as rhizosphere that is subjected to rapid changes. These changes may be due to intense microbial activity influenced by deposits of as much as 20% of the carbon allocated to roots (Freckman and Casewell, 1985). Rainfall and daytime drought can result in fluctuations in salt concentration, pH, osmotic potential, water potential, and soil particle structure in short temporal scales. Over longer temporal scales, on the other hand, the rhizosphere can change due to root growth, interactions with other soil biota, and weathering processes. These complex conditions and biological interactions may influence on disease development or its biocontrol (Deacon, 1991; Rovira, 1965, 1991; Waisel, 1991).

Acknowledgements

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