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Biological Control of Soil Pests by Mixed Application of Entomopathogenic and Fungivorous Nematodes[1]

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Abstract: In greenhouse experiments, massive application of the fungivorous nematode, *Aphelenchus avenae,* in summer at 26–33 C (1×10^5 nematodes/500 cm^3 autoclaved soil) or in autumn at 18–23 C (5×10^4 nematodes/500 cm^3 autoclaved soil) suppressed pre-emergence damping-off of cucumber seedlings due to *Rhizoctonia solani* AG-4 by 67% or 87%, respectively. Application of 2×10^5 *A. avenae* to sterilized soil infested with *R. solani* caused leafminer-like symptom on the cotyledons, which did not occur in mixed inoculations with the entomopathogenic nematode, *Steinernema carpocapsae.* When 1×10^6 *A. avenae* were applied 3 days before inoculation with 100 *Meloidogyne incognita* juveniles, gall numbers on tomato roots were reduced to 50% of controls. Gall numbers also were suppressed by *S. carpocapsae* (str. All). Reduction in gall numbers was no greater with mixed application of *A. avenae* and *S. carpocapsae* than with application of single species, even though twice the number of nematodes were added in the former case. These nematodes were positively attracted to tomato root tips. *Aphelenchus avenae* suppressed infection of the turnip moth, *Agrotis segetum,* but not the common cutworm, *Spodoptera litura,* by *S. carpocapsae.*

Key words: *Aphelenchus avenae,* biological control, *Meloidogyne incognita, Rhizoctonia solani, Steinernema carpocapsae.*

The entomopathogenic nematode *Steinernema carpocapsae* is a promising biological agent for a broad range of soil-inhabiting insect pests. Practical control of insect pests by these nematodes was demonstrated on common cutworm, *Spodoptera litura,* and turnip moth, *Agrotis segetum,* under greenhouse and field conditions (16). Inundative soil application of these nematodes also affected the population densities of native nematodes; free-living nematodes increased, whereas plant-parasitic nematodes decreased (15–17). Bird and Bird (4) clearly demonstrated the same phenomena with *Meloidogyne javanica* and *Steinernema glaseri.* They suggested that penetration of *M. javanica* into roots was suppressed due to competition for space with *S. glaseri,* which also was attracted to the tomato roots.

The fungivorous nematode *Aphelenchus avenae* is ubiquitous in soil. The nematode can be propagated on axenically cultured tissues of carrot and on tobacco and tomato callus; therefore it was regarded as plant-parasitic (2). On the other hand, *A. avenae* failed to parasitize many plants under greenhouse conditions (22). Because *A. avenae* lives on many kinds of soil fungi (24), trials have been conducted to see if this nematode controlled soil-borne pathogenic fungi. The nematode appears to have promise as a biological control agent of certain pathogenic fungi (1,3,6,7,9,13).

This paper reports the efficacy of *A. avenae* as a biological control agent against pre-emergence damping-off of cucumber caused by *Rhizoctonia solani,* the suppressive effect of *A. avenae* and (or) *S. carpocapsae* as nonparasitic, nonpredacious antagonists against *M. incognita* juveniles, and the possibility of simultaneous control of fungal and insect pests and plant-parasitic nematodes by mixed application of these nematodes.

Materials and Methods

Aphelenchus avenae from a spinach field at Kagoshima, Japan, was cultivated on *Botrytis cinerea* growing on wheat bran at 25 C (10). About 30 days after incubation, fourth-stage and adult nematodes were collected by sieving and used in the exper-

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iments. *Steinernema carpocapsae* (str. All) was propagated on chicken offal medium for 2 months at 25 C (19). The infective juveniles were used.

Egg masses of *Meloidogyne incognita* from severely galled roots of tomato were surface sterilized with 1% NaOCl and rinsed in sterilized distilled water three times. Second-stage juveniles (J2) hatched from egg masses were used as inocula.

A virulent strain of *Rhizoctonia solani* AG-4 was grown on autoclaved wheat bran medium (10 g bran plus 15 ml water) at 25 C for 5 days. The freshly colonized medium was used as inoculum for the fungus.

Spodoptera litura and *Agrotis segetum* were reared at 25 C on artificial diet (20) under a photoperiod of 16 hours light and 8 hours dark. Sixth-instar larvae of the insects were used for experiments.

Control of pre-emergence damping-off by A. avenae: Autoclaved sandy loam soil (500 cm^3) containing 1 g inoculum of *R. solani* plus 0, 1×10^4, 3×10^4, 6×10^4, 1×10^5, or 2×10^5 *A. avenae* was added to each of five replicate clay pots. Control pots received neither fungus nor nematodes. The pots were placed in a greenhouse. After 2 weeks, three cucumber (*Cucumis sativus* L. cv. Soeyoh) seeds were sown in each pot. Four weeks later, the surviving plants in each pot were counted. The experiment was performed once in late August (26–33 C) and again in October (18–23 C).

Effect of mixed inoculation with A. avenae and (or) S. carpocapsae and (or) M. incognita on pre-emergence damping-off: The following soil treatments were established: *R. solani*; *R. solani* and *M. incognita*; *R. solani* and *A. avenae*; *R. solani, A. avenae,* and *S. carpocapsae*; *R. solani, A. avenae, S. carpocapsae,* and *M. incognita*; and a sterilized soil control. Inoculum levels were 1 g *R. solani,* 2×10^5 *A. avenae,* 5×10^5 *S. carpocapsae,* and 1×10^2 *M. incognita* per pot. Procedures for preparation of pots, sowing of seeds, and growing of plants were the same as for the previous experiment. Pots were maintained at 18–23 C, and seedling survival and condition were assessed 30 days after sowing. The experiment was performed once.

Aphelenchus avenae and S. carpocapsae as antagonists of M. incognita: One tomato seedling (7–8 cm high) was planted in each of 200 plastic pots containing 250 cm^3 autoclaved sandy loam soil. At the same time, two glass tubes (0.5 cm d, 5 cm long) were inserted in the soil 2 cm deep, on opposite sides and 2 cm from the plant stem. Thirteen days later, 100 J2 of *M. incognita* in 10 ml water (5 ml per tube) were added. A range of *A. avenae* and (or) *S. carpocapsae* (10^2 to 5×10^6 in 10 ml water) was added to the tubes either 3 days before, at the same time as, or 3 days after the addition of *M. incognita.* Ten replications of the following four treatments were maintained: *M. incognita* alone as controls; *M. incognita* and *A. avenae*; *M. incognita* and *S. carpocapsae*; and *M. incognita, A. avenae,* and *S. carpocapsae.* For the last treatment, the total number of nematodes was twice that for other treatments at each dose level. The roots of five replicate plants per treatment were stained (5) and galls were counted 15 and 30 days after inoculation with *M. incognita.*

Orientation of S. carpocapsae and A. avenae to tomato root tips: Tomato seeds were sonicated in 2.5% NaOCl for 5 minutes, then washed several times with sterile water before placement on 0.75% agar with Gamborg's B-5 medium (14) in petri dishes (9 cm d). Seeds were placed in light after germination. When seedlings had a radicle ca. 3 cm long and cotyledons were green, ca. 5×10^3 *A. avenae* and (or) 5×10^3 *S. carpocapsae* concentrated on a 0.45-μm-pore membrane filter were brushed onto an inoculation zone (1 cm d) 1 cm from the root tip (12). Five replications were maintained at room temperature for each treatment. The behavior of the nematodes was qualitatively assessed for 1 hour. This experiment was conducted four times.

Effect of A. avenae on infectivity of S. carpocapsae: A nematode suspension (0.4 ml) containing 50 *S. carpocapsae* and 0, 50, 500, or 5,000 *A. avenae* was added to a circular filter paper (5 cm d) in a plastic petri dish

(5.3 cm d). In an additional treatment, the suspension contained no *S. carpocapsae* and 5,000 *A. avenae.* One last-instar larva of *S. litura* or *A. segetum* was placed in each dish and incubated at 25 C. There were 20 dishes per level of *S. carpocapsae--A. avenae.* Mortality of insects was determined by touch response at 8-hour intervals from 24 to 120 hours. The experiment was performed once.

Effect of S. carpocapsae and A. avenae on pre-emergence damping-off: A nematode suspension (50 ml) containing 1 × 10^4 *A. avenae* and 0, 1 × 10^4, 1 × 10^5, or 1 × 10^6 *S. carpocapsae* was sprayed on the colonized medium (1 g wheat bran) of *R. solani,* which was evenly spread on the top of 450 cm^3 autoclaved sandy loam soil in a clay pot. Immediately after the addition of the nematodes, 50 cm^3 soil was layered on top so that the final volume was 500 cm^3. Pots were wrapped in polythene bags to prevent desiccation and placed in a greenhouse at 18–25 C. There were five replications per level of *A. avenae–S. carpocapsae.* Emergence of seedlings was followed for 10 days. This experiment was performed once.

Results

Control of pre-emergence damping-off by A. avenae: Pre-emergence damping-off of cucumber due to *R. solani* was reduced significantly by *A. avenae* (Table 1). No post-emergence damping-off was observed during the experiment. In summer, when the greenhouse temperature ranged from 26 to 33 C, no seedlings emerged in soil inoculated with *R. solani* alone, whereas 33–80% emerged with application of *A. avenae.* In fall, when the temperature ranged from 18 to 23 C, a low percentage of seedlings emerged in soil inoculated with *R. solani* alone. Application of nematodes again increased emergence; however, inoculation with 2 × 10^5 or more nematodes per pot caused a leafminer-like symptom on the cotyledons of young cucumber seedlings and a slight decrease in emergence. No retardation in the further growth of these plants was observed. The symptom on the cotyledons did not occur in the summer experiment.

Table 1. Suppression of pre-emergence damping-off of cucumber seedlings due to *Rhizoctonia solani* AG-4 by *Aphelenchus avenae.*

Inoculum level†	Seedling emergence (%)‡	Seedlings (N) alive/dead
Experiment 1 (26–33 C)		
0	0 ± 0	0/15 a
1 × 10^4	33 ± 26	5/10 b
3 × 10^4	33 ± 26	5/10 b
6 × 10^4	40 ± 45	6/9 b
1 × 10^5	66 ± 40	10/5 b
2 × 10^5	80 ± 8	12/3 c
Control	100 ± 0	15/0 c
Experiment 2 (18–23 C)		
0	13 ± 8	2/13 a
5 × 10^4	86 ± 8	13/2 bc
1 × 10^5	86 ± 8	13/2 bc
2 × 10^5	80 ± 8	12/3 bc
5 × 10^5	66 ± 26	10/5 b
1 × 10^6	73 ± 5	11/4 bc
Control	100 ± 0	15/0 c

Number of alive/dead followed by the same letter in each experiment are not different ($P = 0.05$).
† Numbers of *A. avenae* added per pot containing 1 g wheat bran medium colonized by *R. solani.* Control pots received neither nematodes nor fungus.
‡ Values are means ± SD of five replicate pots.

Effect of A. avenae and (or) S. carpocapsae and (or) M. incognita on pre-emergence damping-off: Cucumber seedlings failed to emerge in soil inoculated with *R. solani* alone or *R. solani* plus *M. incognita.* All seedlings emerged in soil inoculated with *R. solani* plus *A. avenae, R. solani* plus *A. avenae* and *S. carpocapsae,* and in uninoculated soil. In soil inoculated with *R. solani* plus *A. avenae, S. carpocapsae,* and *M. incognita,* 80% of the seedlings emerged. A leafminer-like symptom of the cotyledons was caused by inoculation with *R. solani* and *A. avenae* but was not observed in the other treatments. Seedling height was less ($P = 0.05$) in the presence of *R. solani* and *A. avenae* (23.1 ± 1.3 cm) than in the uninoculated control (26.3 ± 1.3 cm). Seedling height in the other treatments was not different from the control.

Aphelenchus avenae and S. carpocapsae as antagonists of M. incognita: Numbers of *M. incognita*-induced galls in tomato roots decreased with application of *A. avenae* or *S.*

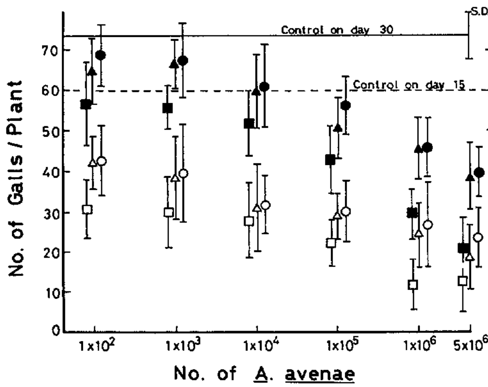

FIG. 1. Numbers of *Meloidogyne incognita*-induced galls on tomato as affected by *Aphelenchus avenae*. *Aphelenchus avenae* was applied 3 days before (square), simultaneously with (triangle), or 3 days after (circle) inoculation with 100 *M. incognita* second-stage juveniles. White and black symbols indicate the number (mean ± SD of five replicates) of galls per plant 15 and 30 days, respectively, after inoculation with *M. incognita*. Horizontal lines indicate numbers of galls after 15 and 30 days on plants inoculated with *M. incognita* only.

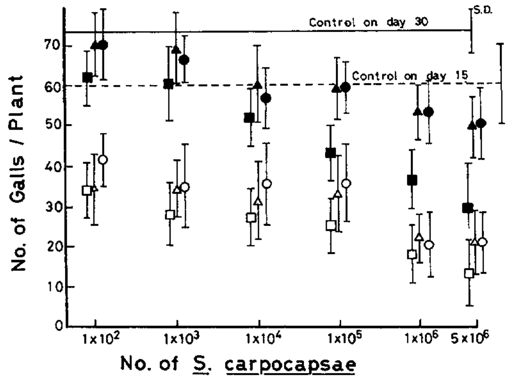

FIG. 2. Numbers of *Meloidogyne incognita*-induced galls on tomato as affected by *Steinernema carpocapsae*. *Steinernema carpocapsae* was applied 3 days before (square), simultaneously with (triangle), or 3 days after (circle) inoculation with 100 *M. incognita* second-stage juveniles. White and black symbols indicate the number (mean ± SD of five replicates) of galls per plant 15 and 30 days, respectively, after inoculation with *M. incognita*. Horizontal lines indicate numbers of galls after 15 and 30 days on plants inoculated with *M. incognita* only.

carpocapsae (Figs. 1, 2). Application of these nematodes 3 days before *M. incognita* tended to reduce gall formation more than did simultaneous application or application 3 days after; however, the timing of inoculation was not significant ($P > 0.05$), except at day 30 and with application of 1×10^5 or more nematodes 3 days before addition of *M. incognita*. Although there tended to be more reduction of gall formation with *A. avenae* than with *S. carpocapsae*, the difference was not significant ($P > 0.05$). Despite the twofold increase in total nematodes at each dosage level, mixed application of these nematodes had less effect than did the single application of either nematode (Fig. 3).

Orientation of A. avenae and S. carpocapsae to root tips: A. avenae moved rapidly to the root tips on agar plates and swarmed around the meristematic region (Fig. 4). In contrast, 40–50% of *S. carpocapsae* juveniles stayed in the inoculation zone and became motionless within 1 hour after inoculation; the remainder moved to the root tips and to the differentiation zone. When *S. carpocapsae* was placed on the root tip, very few nematodes dispersed beyond the

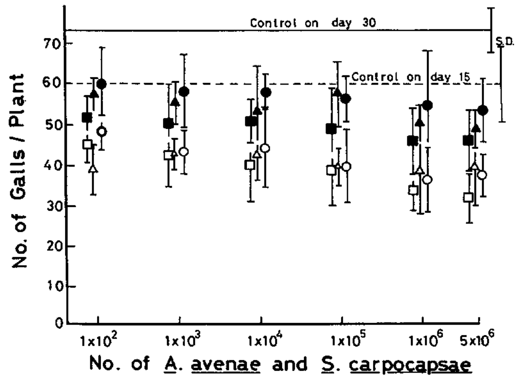

FIG. 3. Numbers of *Meloidogyne incognita*-induced galls on tomato as affected by mixed application of *Aphelenchus avenae* and *Steinernema carpocapsae*. Each nematode species was applied with the number of individuals on abscissa. Nematodes were applied 3 days before (square), simultaneously with (triangle), or 3 days after (circle) inoculation with 100 second-stage juveniles of *M. incognita*. White and black symbols indicate the number (mean ± SD of five replicates) of galls per plant 15 and 30 days, respectively, after inoculation with *M. incognita*. Horizontal lines indicate numbers of galls after 15 and 30 days on plants inoculated with *M. incognita* only. Regressions for day 30 and pre-application data were $Y = -7.6X + 62.6$ ($r = -0.9482$) for *A. avenae*, $Y = -6.2X + 61.2$ ($r = -0.9731$) for *S. carpocapsae*, and $Y = -1.1X + 51.8$ ($r = -0.7527$) for the mixture, where Y is the number of galls per plant and X is the number of applied nematodes on logarithmic scale.

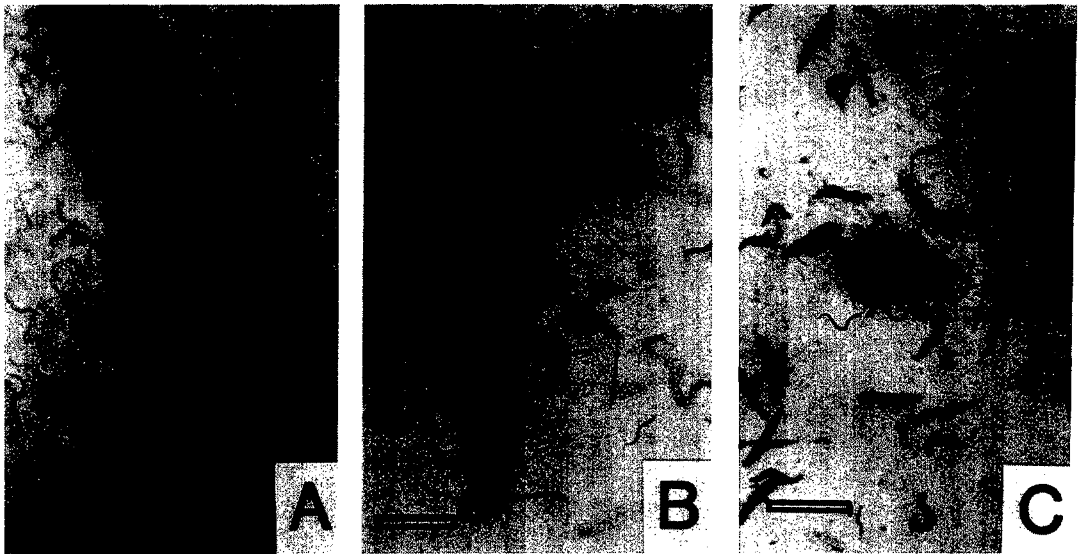

FIG. 4. Attraction of nematodes to tomato root on agar. A) *Aphelenchus avenae* swarming around root tip. B) *Steinernema carpocapsae* clinging onto root surface. C) Clusters of *S. carpocapsae* induced by mixed inoculation with *A. avenae*. Scale bars = 1 mm.

inoculation zone. When added together, *A. avenae* did not aggregate at the root tip but dispersed in the agar plate; *S. carpocapsae* formed clusters in the inoculation zone.

Infectivity of S. carpocapsae as affected by A. avenae: Infection of *S. litura* by *S. carpocapsae* was unaffected by *A. avenae,* whereas infection of *A. segetum* was decreased (P = 0.05) by high number of *A. avenae* (Table 2).

Effect of S. carpocapsae and A. avenae on pre-emergence damping-off: No seedlings of cucumber emerged in the soil inoculated with *R. solani* alone. Application of 1×10^4 *A. avenae* without *S. carpocapsae* resulted in 100% emergence 11 days after sowing. Addition of *S. carpocapsae* resulted in 100% emergence on days 8, 8, and 7 for 1×10^4, 1×10^5, and 1×10^6 *S. carpocapsae,* respectively. In the latter two treatments, emergence occurred sooner ($P = 0.05$) than with *A. avenae* alone.

DISCUSSION

In the present study, *A. avenae* substantially suppressed *R. solani*-induced damping-off of cucumber. Suppression was greater at 18–23 C than at 26–33 C. We suggest that this is due to the higher temperature optimum for *R. solani* (21) than

TABLE 2. Infection (% mortality) of *Spodoptera litura* and *Agrotis segetum* by *Steinernema carpocapsae* (str. All) as affected by *Aphelenchus avenae*.

Inoculum/insect			*S. litura*			*A. segetum*			
S. carpocapsae	+	*A. avenae*	48 hr	72 hr	96 hr	48 hr	72 hr	96 hr	120 hr
0	+	0	0 a	0 a	0 a	0 a	0 a	0 a	0 a
0	+	5,000	0 a	0 a	0 a	0 a	0 a	0 a	0 a
50	+	0	85 b	95 b	100 b	50 b	80 d	90 c	90 c
50	+	50	95 b	100 c		40 b	60 cd	80 c	90 c
50	+	500	95 b	100 c		10 a	40 bc	60 bc	60 bc
50	+	5,000	95 b	100 c		0 a	20 b	40 b	60 bc

Each treatment had 20 petri dishes each containing one last-instar larva with artificial food.
Values followed by the same letter within a column are not significantly different (P = 0.05) based on χ^2-test.

for *A. avenae.* Because the experiments were not repeated or conducted at the same time, additional research is needed on the effect of temperature. A leafminer-like symptom on cotyledons of cucumber seedlings was induced by massive application of *A. avenae* in sterilized soil (7) and by combined inoculation of *A. avenae* and *R. solani* in the present study. This symptom did not occur when both *A. avenae* and *S. carpocapsae* were added to *R. solani*-infested soil. Although *A. avenae* appears to have some detrimental effects on the host plant, we have not observed substantial damage to plants under field conditions (unpubl.).

That plant-parasitic nematodes were suppressed by inundative application of entomopathogenic nematodes to soil (4,15,17) was confirmed by the present experiments. Bird and Bird (4) reported the attraction of *S. glaseri* to tomato root tips on agar plates and a decrease in the numbers and reproductive capacity of *M. javanica* when *S. glaseri* was applied in pot experiments. They suggested that *S. glaseri* was attracted to CO_2 from the root tips and that *M. javanica* juveniles failed to compete for root surface required for penetration because *S. glaseri* is much larger and more active than *M. javanica.* We also observed that *A. avenae* and *S. carpocapsae* were positively attracted to, and aggregated around, root tips. Both *A. avenae* and *S. carpocapsae* suppressed gall number on tomato roots. Mixed application of these nematodes was expected to cause greater reduction in gall formation than single application, but the opposite was true. This suggests an antagonistic interaction between *A. avenae* and *S. carpocapsae.* The mechanism is unknown, but contact stimulation may cause nematodes to disperse as appeared to occur when both nematodes were added to roots on agar plates in the present study. Infection of *A. segetum* but not *S. litura* by *S. carpocapsae* was decreased by *A. avenae,* perhaps because *A. segetum* is more resistant than is *S. litura* to *S. carpocapsae* (23). In contrast, *S. carpocapsae* did not lessen the suppression of pre-emergence damping-off by *A. avenae.*

Commercial application of *A. avenae* at the rates used in the present experiments will be possible. *Aphelenchus avenae* can be mass produced on industrial by-products such as wheat bran, beet pulp, bagasse, brewer's grain (malt barley), or combinations of these materials (8,10). One kilogram (dry weight) of these materials will produce 200–300 million nematodes (8). In a field trial, complete control of *R. solani*-induced damping-off of spinach was achieved by application of *A. avenae* together with its culture substrates (beet pulp plus brewer's grain) as a basal dressing at the rate of 1 kg/2 m of plant row (18). Massive application of entomopathogenic nematodes (0.5–1 million/m^2) is also commercially possible.

Although further basic studies are needed on the interaction between entomopathogenic and fungivorous nematodes, mixed application of these beneficial nematodes may provide simultaneous control of soil insect pests, plant-parasitic fungi, and plant-parasitic nematodes. These nematodes may also be used with certain chemical pesticides (11,16).

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