Dry Mycelium of *Penicillium chrysogenum* Protects Cucumber and Tomato Plants against the Root-knot Nematode *Meloidogyne javanica*

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Incorporation into soil of dry mycelium of *Penicillium chrysogenum*, a waste product of the pharmacological industry, enhanced plant growth and reduced root galling caused by the root-knot nematode *Meloidogyne javanica* in cucumber and tomato plants. Incorporation into sandy loam soil in pots of dry mycelium at a concentration of 0.25% (w/w) resulted in complete protection of cucumber plants from the nematode. The number of juveniles recovered from soils containing dry mycelium was greatly reduced even at a concentration of 0.1% (w/w). In microplot studies conducted at two sites in two seasons, with three or four doses, dry mycelium caused a dose-dependent reduction in root galling index (GI) and promotion of plant growth of cucumber and tomato plants. In *in vitro* studies, the water extract of dry mycelium immobilized nematode juveniles and reduced the egg hatching rate, but these effects were partly reversible after a rinse in water. Soil-drenching of cucumber and tomato seedlings with water extract of dry mycelium did not reduce GI or number of root-invading juveniles. The results show that dry mycelium promotes plant growth and protects plants against nematode infection. Protection, however, does not operate *via* induced resistance.

KEY WORDS: *Meloidogyne javanica*; *Penicillium chrysogenum*; root-knot nematode; soil amendment.

INTRODUCTION

Plant-parasitic nematodes have been controlled mainly by chemical nematicides, but several effective nematicides and fumigants have been withdrawn from the market because of their deleterious effects on human health and the environment. In particular, the reduction in use and phasing out of methyl bromide in recent years have made nematode control more difficult (18). Cultural practices such as use of resistant varieties, crop rotations and soil amendments are often integrated into nematode control strategies. Organic soil amendments, especially those with low C/N ratios, have been reported to have nematicidal effects (25). Crab shell-based products and chitin, which is found in the exoskeletons of insects and crustaceans, and in cell walls of certain fungi as polymers, have been studied extensively for their effects on nematode and fungal pathogen control (2,26,27). These amendments in soil are subjected to microbial decomposition, and several volatiles with nematicidal activity, especially ammonia, are released (20,26).

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Besides the direct nematicidal effect of these volatiles in earlier decomposition processes, the build-up of antagonistic microorganisms is also thought to be involved in nematode control by organic amendments (12,26). However, this approach is usually limited due to the large quantity of amendments required for control and irreproducible efficiencies, which probably result from soil conditions (29). Enhancement of natural plant-defense mechanisms is another control strategy. Several compounds are known to elicit plant defense responses, which induce local and/or systemic resistance and protect plants from a wide range of pathogens. Salicylic acid, the most investigated compound, induces resistance in plants against several pathogens (28). β-aminobutyric acid, a natural non-protein amino acid, has been reported to induce resistance to a wide range of plant pathogens, including nematodes (7). Microorganisms and compounds of microbial origin have also been found to induce defense responses and/or resistance in plants toward pathogens (5,14,23,30). For example, oligomers of chitosan, which may be released by plant chitosanase from invading fungal pathogens, have been used to induce resistance against fungi (3,4). Dry mycelium (DM) of *Penicillium chrysogenum* from the antibiotics industry has been reported to induce resistance against *Verticillium dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum* in cotton and against *F. oxysporum* f.sp. *melonis* in melon (8-10). Increases in free L-proline in melon, and peroxidase activities in melon and cotton, have been induced after treatments with DM (8,10).

As compared with fungal or bacterial pathogens, induced resistance against plant-parasitic nematodes has not been studied thoroughly. β-aminobutyric acid (BABA) has been shown to induce resistance against certain root-knot and cyst nematodes (7,21,22). Other compounds, such as salicylic acid and lipopolysaccharides, and microorganisms including avirulent nematodes and rhizobacteria, have been reported to increase resistance against nematodes (13,16,19,24). In the present study, the direct and indirect effects of DM of *P. chrysogenum* on the root-knot nematode *Meloidogyne javanica* were investigated in tomato and cucumber plants.

MATERIALS AND METHODS

Dry mycelium and extract DM of *P. chrysogenum* was provided by Biochemie Ltd. (Kundl, Austria). This nonviable fungal biomass was dried for 4 h at 110°C by the manufacturer and contains no residues of penicillin. DM contains ~90% organic matter, 7% N, 1% P and 2% K. Water extract of DM (DME) was prepared as follows: 100 g of DM was mixed with tap water to make 1000 ml suspension. The slurry was stirred overnight at 4°C, and filtered through Whatman No. 1 filter paper. The filtrate was centrifuged at 5000 RCF (relative centrifugal force) for 10 min and filtered through a 0.2-μm filter. This water extract (10% DME) had a pH of 3.5, which was modified to pH 7.0 using 1.0 N NaOH solution before filtration through a 0.2-μm filter.

Nematode Eggs of *M. javanica* were extracted from nematode-infected tomato (*Lycopersicon esculentum* cv. 'Hazera-144') roots with sodium hypochlorite solution (15). Second-stage juveniles (J2) of the nematode were collected daily from eggs, spread on a 30-μm sieve, and stored at 15°C. Juveniles aged for less than 3 days were used in the *in vitro* and pot experiments. J2 and eggs for *in vitro* experiments were washed with sterile water several times.

***In vitro* experiments** *DME:* Approximately 100 J2 or eggs of *M. javanica* in 10 μl of water were placed into 500 μl of diluted (1%, 2%, 4% and 8%) and undiluted (10%) DMEs containing 100 μg ml^{-1} rifampicin for 2 and 3 days at 27°C, respectively. The number of immobilized J2 and emerged J2 were counted after incubation, and DMEs were replaced with tap water. The number of immobilized J2 and emerged J2 were counted again after 2 and 4 days in the tap water, respectively. Each treatment had four replicates. Tap water with rifampicin was served as control. This experiment was repeated with pH-modified (7.0) DME.

DM in soil: Approximately 150 J2 in 2 ml of water were added to 10 g of dry non-sterilized dune sand (pH 9.2) containing (w/w) 0.1% (pH 8.7), 0.2% (pH 8.3), 0.4% (pH 7.8), 0.8% (pH 7.4) and 1.6% (pH 7.0) DM in 25-ml glass bottles for 7 days at 27°C. Sand pH was measured after stirring 10 g of sand in 20 ml of distilled water. Sand without DM served as a control. Live juveniles were recovered from the sand through a sieve (60 μm) by the Baermann tray method (1) and counted. This experiment was repeated twice with five replicates.

Pot experiments *Drenching:* Tomato seedlings (30 days old) grown on 200 g sand in 150 ml plastic pots were soil drenched with 5 ml of 0.5%, 1.0%, 2.0%, 4.0%, 8.0% and 10% DME. Two days after drenching, the plants were inoculated with *ca* 500 J2, and kept in a growth chamber at 27± 2°C with 13 h light per day. Plants drenched with water served as controls. The galling index (GI) of the roots was assessed 10 days after inoculation using a 0 to 5 rating system: 0, no infection; 1, 1–20%; 2, 21–40%; 3, 41–60%; 4, 61–80%; and 5, 81–100% of roots galled (11). In a similar experiment, germinating cucumber seeds were planted in soil in 75-ml pots and treated with 2 ml of 0.5%, 1.0%, 2.0%, 4.0%, 8.0% and 10% DME. The plants were inoculated with *ca* 200 J2 2 days after planting. Plants drenched with water served as controls. The number of nematodes in roots was counted with the aid of a dissecting microscope 7 days after inoculation, and following staining with acid fuchsin (6). These experiments had six replicates per treatment.

Incorporation: DM was mixed with sand (600 g) at doses of 0.1%, 0.2% and 0.4% (w/w) in 500-ml pots. The soils were then inoculated with *ca* 2000 J2. Tomato seedlings were planted in the soils 7 days after inoculation. The GIs of the root systems were recorded 24 days after planting. Plants grown in untreated sand served as a control. Each treatment had six replicates.

DM powder was mixed with sand at doses of 0, 0.25%, 0.5% and 1% (w/w). Sand mixtures were placed into 150-ml pots and two cucumber seeds were sown in each pot. Ten pots (with 20 seeds) were used with each sand mixture. One day after sowing, 1 ml of water containing 500 J2 larvae of *M. javanica* was injected into four 1-cm-deep holes in each pot with a pipette. Twenty days after sowing, plants were uprooted, washed, weighed (total plant), and their root GI was recorded.

Shade-house experiments Four experiments were conducted during 2000–2002 in two shade houses naturally infested with *M. javanica*. Three experiments were conducted on the campus of Bar-Ilan University, and one on the experimental farm of the university, all laid out in a randomized block design with four replicates. DM was spread over the microplots and incorporated into the soil to a depth of 10–15 cm. Plants were drip irrigated with tap water with no fertilizers added during the growing period. At various time intervals after sowing, plants were uprooted from soil and washed with tap water; galling on the root was

recorded visually (0–5 scale) and the shoot was weighed.

A: There were four 20 × 0.5 m beds (soil pH 6.1) in a shade house on the campus. DM was applied to 2 × 0.5 m subplots at doses of 0, 0.25, 0.5, 1 and 1.5 kg m^{-2}. Thereafter, two beds were soil-solarized by plastic mulch for 18 days in July 2000 and two beds were left unsolarized. Plastic mulch was removed and cucumber seeds (cv. 'Kafir') were sown (20 seeds per plot). Plants were uprooted 45 days after sowing, their roots were washed with tap water and evaluated for GI and shoot weight.

B: There were eight 20 × 0.5 m beds in a shade house. DM was applied in late March 2002 to 2 × 0.5 m subplots at doses of 0, 0.125, 0.25 and 0.5 kg m^{-2} in a completely randomized block design, with 24 replicate subplots per dose. Cucumber seeds were sown (20 seeds per plot) at 10 days after DM application. Plants were uprooted for analysis (GI and fresh shoot weight) at 21, 40 and 48 days after sowing. For each sampling time, eight subplots per dose were used. Two days after the last harvest (mid May 2002), subplots were again amended with DM, each with the same dose as in the previous experiment. Tomato seedlings (cv. 'Roter Gnom') were planted one day later, five plants per subplot, totaling 24 subplots per treatment dose. At 53 days after planting (early July 2002), plants were uprooted from soil, washed, and the GI and shoot fresh weight were determined.

C: Four beds (soil pH 6.1) in a shade house on the experimental farm were divided into 20 subplots of 1 × 0.5 m each. DM was applied to the soil on May 22, 2002, at doses of 0, 0.25, 0.5 and 1 kg m^{-2}; cucumber seeds (ten seeds per plot) were sown one day later. Thirty days after sowing, plants were uprooted, washed with water, and the GI and shoot fresh weight were determined.

Data analysis In all experiments data were statistically analyzed using Duncan's Multiple Range Test to identify differences at the 5% probability level. Data presented as percentages were transformed by inverse sine transformation ($\sin^{-1}\sqrt{x}$) before analysis.

RESULTS

***In vitro* experiments** DM incorporated into the sand seemed to kill the nematode J2 (Table 1). The number of recovered J2s was significantly reduced even at a concentration of 0.1% DM (w/w). The number of J2s was reduced by exposure of eggs to the pH-unmodified DMEs at concentrations of 8% and 10% (Table 2). However, there was no difference in number of emerged J2 during another 4 days after a rinse with water. The pH-modified (pH 7.0) DMEs had no effect on J2 hatching even during the exposure to the extract. Nematode J2 exposed to high concentrations of pH-unmodified (pH 3.5) DME were immobilized but some recovered after a rinse in tap water (Table 3). DMEs with pH 7.0 had no effect on J2 mobility.

TABLE 1. Numbers of second-stage juveniles of *Meloidogyne javanica* recovered from soils containing various concentrations (%, w/w) of *Penicillium chrysogenum* dry mycelium (DM; *in vitro* experiments)

DM (%)	0	0.1	0.2	0.4	0.8	1.6
Exp. 1	81.8 a[z]	nt[y]	20.4 b	1.8 c	0 c	0 c
Exp. 2	83.4 a	22.4 bc	11.5 c	0.4 d	0 d	nt

[z]Within columns, figures followed by the same letter do not differ significantly at P=0.05 (Duncan's MRT).
[y]nt, not tested.

TABLE 2. Effect of water extracts of *Penicillium chrysogenum* dry mycelium (DME) on hatching of *Meloidogyne javanica* eggs

DME (%)	0	1	2	4	8	10
			pH 3.5			
Hatching (%)	27.3 a[z]	28.5 a	30.0 a	31.5 a	16.0 b	1.0 c
After rinse	46.8 a	43.0 a	41.3 a	42.4 a	36.5 a	36.5 a
			pH 7.0			
Hatching	21.3 a	26.3 a	20.3 a	28.0 a	20.0 a	17.8 a
After rinse	43.8 a	40.8 a	38.5 a	47.8 a	38.5 a	41.8 a

Hatching percentages were calculated after 3 days of exposure of eggs to the extracts and after another 4 days in tap water. The extracts were prepared from the stock extract (10%, pH 3.5) by dilution with tap water. Modification of pH (7.0) was done with NaOH.

[z] Within columns, figures followed by the same letter do not differ significantly at P=0.05 (Duncan's MRT) after transformation by inverse sine [$\sin^{-1}\sqrt{x}$]).

TABLE 3. Effect of water extracts of *Penicillium chrysogenum* dry mycelium (DME) on mobility (%) of *Meloidogyne javanica* second-stage juveniles

DME (%)	0	1	2	4	8	10
			pH 3.5			
Mobility (%)	95.8 a[z]	90.1 ab	82.6 b	18.0 c	7.3 cd	0 d
After rinse	97.6 a	96.5 a	97.6 a	90.8 a	70.0 b	60.5 b
			pH 7.0			
Mobility (%)	92.9 a	91.1 a	89.7 a	85.5 a	86.5 a	87.3 a
After rinse	96.1 a	96.4 a	97.0 a	96.1 a	95.2 a	94.4 a

Percentages of mobile juveniles were calculated after 3 days of exposure of juveniles to the extracts and after another 2 days in tap water. The extracts were prepared from the stock extract (10%, pH 3.5) by dilution with tap water. Modification of pH (7.0) was done with NaOH.

[z] Within columns, figures followed by a common letter do not differ significantly at P=0.05 (Duncan's MRT) after transformation by inverse sine [$\sin^{-1}\sqrt{x}$]).

TABLE 4. Effect of soil amendment with *Penicillium chrysogenum* dry mycelium (DM) on plant growth and root infection of cucumber and tomato with *Meloidogyne javanica* in naturally infested soil in a shade house

DM amendment (kg m^{-2})	Cucumber				Tomato			
	Days after sowing/planting							
	21 d		40 d		48 d		53 d	
	SFW[z] (g plant^{-1})	GI[y]	SFW (g plant^{-1})	GI	SFW (g plant^{-1})	GI	SFW (g plant^{-1})	GI
0	2.7 b[x]	1.7 a	22 c	1.8 a	28 c	1.5 a	237 b	3.0 a
0.125	3.5 ab	1.5 a	56 b	1.4 ab	86 b	1.1 a	593 a	1.8 b
0.25	4.0 a	0.7 b	86 a	0.6 b	133 a	0.7 b	656 a	1.8 b
0.5	4.2 a	0.4 b	99 a	0.9 b	134 a	0.7 b	648 a	1.4 b

[z] SFW = shoot fresh weight.

[y] GI = galling index, 0–5 visual scale.

[x] Within columns, figures followed by a common letter do not differ significantly at P=0.05 (Duncan's MRT).

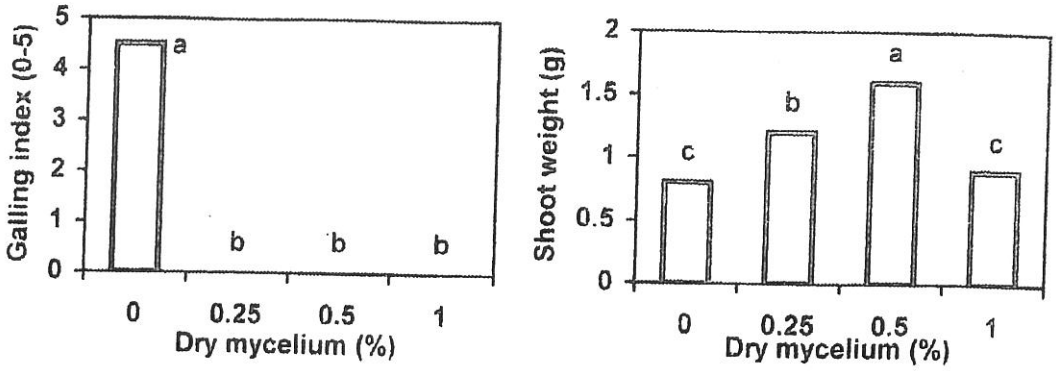

Fig. 1. Effect of *Penicillium chrysogenum* dry mycelium incorporated (%, w/w) into *Meloidogyne javanica*-infested soil on root galling index (left) and fresh shoot weight (right) of cucumber plants in pots. Values in columns marked with the same letter do not differ significantly (P=0.05) according to Duncan's MRT.

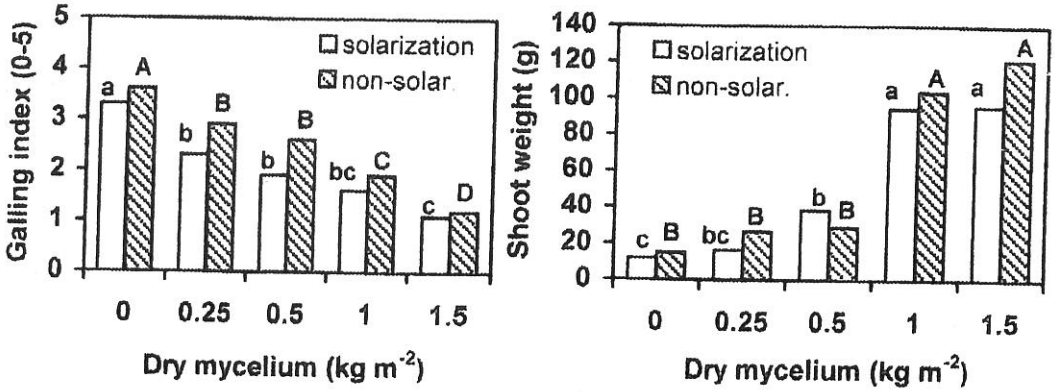

Fig. 2. Effect of *Penicillium chrysogenum* dry mycelium incorporated into *Meloidogyne javanica*-infested soil, with or without soil solarization, on root galling index (left) and fresh shoot weight (right) of cucumber plants in a shade house. Values in columns marked with a common letter do not differ significantly (P=0.05) according to Duncan's MRT.

Pot experiments *Drenching*: There were no differences in GIs between tomato plants treated with DME extracts before nematode inoculation and control plants (data not shown). In the experiment with cucumber seedlings, there was also no significant difference in the number of J2 in the root systems between seedlings soil-drenched with 0.5–10% DME and untreated seedlings (data not shown).

Incorporation: The GI of tomato plants grown in soil treated with DM at a concentration of 0.4% was 2.7, whereas a significantly higher (P<0.05) index was recorded in the control plants (GI= 4.5). Incorporation of DM at concentrations of 0.1% and 0.2% did not reduce the GI (4.8 and 4.7, respectively). In the experiment with cucumber, incorporation of DM reduced the root GI of treated plants as compared with that of the control plants: whereas the GI in roots of control plants (DM-free) was 4.5±0.9, that in all treated plants was 0 (Fig. 1). DM also significantly enhanced plant development at 0.25% and 0.5%, but was inhibitory to plant development at 1% (Fig. 1).

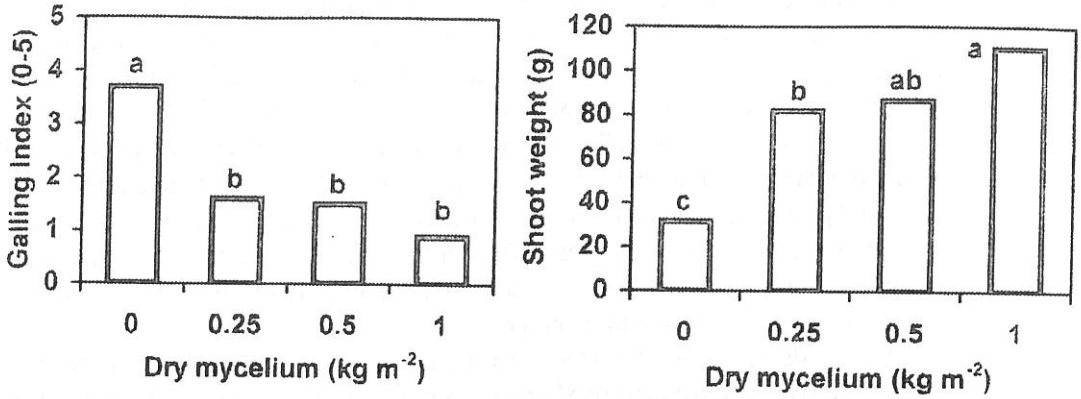

Fig. 3. Effect of *Penicillium chrysogenum* dry mycelium incorporated into *Meloidogyne javanica*-infested soil on root galling index (left) and fresh shoot weight (right) of cucumber plants in a shade house. Values in columns marked with a common letter do not differ significantly (P=0.05) according to Duncan's MRT.

Shade-house experiments

A: Results presented in Figure 2 show a significant decrease in GI and corresponding increase in shoot fresh weight in DM-treated plots. Figure 2 also shows that soil solarization enhanced the control efficacy of DM. Protection against disease in unsolarized plots treated with 0.25, 0.5, 1 and 1.5 kg m^{-2} of DM was 20%, 28%, 47% and 67%, respectively, whereas in solarized plots it was 30%, 42%, 52% and 67%, respectively. The impact of DM on shoot biomass was strong, significant, and dose-dependent. At the highest dose of DM, biomass increased by 710–748% relative to DM-unamended plots. Shoot biomass was somewhat lower in solarized plots than in unsolarized plots.

B: Results showed that amending DM to soil naturally infested with *M. javanica* significantly reduced root galling and significantly enhanced plant shoot development in both cucumber and tomato (Table 4). Root-knot development in cucumber plants growing in control subplots ranged between 1.5 and 1.8 GI. Amending the soil with a low dose of DM (0.125 kg m^{-2}) had a significant, positive effect on shoot growth but not on disease development. Higher doses of DM, 0.25 or 0.5 kg m^2, significantly reduced galling by a mean of 60%. Shoot fresh weight at 48 days increased, relative to control, by 207%, 375% and 378% with DM of 0.125, 0.25 and 0.5 kg m^{-2}, respectively (Table 4). Tomato, as a following crop, exhibited a stronger infection by the nematode, relative to cucumber (Table 4). Galling index in control plants reached a value of 3.0. DM significantly reduced galling by 40%, 40% and 53% at doses of 0.125, 0.25 and 0.5 kg m^{-2}, respectively. Shoot growth of tomato was enhanced, relative to controls, by 150%, 176% and 173% with DM doses of 0.125, 0.25 and 0.5 kg m^{-2}, respectively (Table 4).

C: Results showed that plant growth was significantly enhanced and GI was significantly reduced by DM treatments (Fig. 3). Thus, DM of 0.25, 0.5 and 1.0 kg m^{-2} reduced the GI relative to control plants by 58%, 60% and 74%, respectively, and increased shoot fresh weight by 159%, 176% and 251%, respectively.

DISCUSSION

Our earlier studies showed that DM and its water extract, DME, were capable of inducing resistance against fungal wilt diseases in melon and cotton, while having no

antifungal activity *in vitro* (8-10). Both DM and DME applied to soil enhanced peroxidase activity and L-proline accumulation in root, hypocotyls and even leaves of treated plants, suggesting a systemic action (8-10). Here, DM also reduced damage caused by the root-knot nematode on cucumber and tomato plants. DM was highly protective in pots and partially effective in naturally infested soils in shade houses. In pots, complete control of the nematode on cucumber was observed with 0.25% DM. In shade-house experiments, maximal protection ranged between 67% and 74%, with 0.25 kg m^{-2} (2.5 t ha^{-1}) providing a mean of 42% control. Combining DM with soil solarization enhanced, to a certain extent, nematode control, probably due to the elevated soil temperature and slow emission of toxic volatiles, probably ammonia, from DM.

The mode of action of DM against nematode infection is not clear. Nematode damage reduction may be attributed partly to a direct effect (low pH) of DM or to effects resulting from DM decomposition in soil. Although the pH of the sand which received high concentrations of DM was 7.0–7.4, pH of a microenvironment in the sand, such as water films where the nematodes exist, may be sufficiently lower to kill or immobilize them. This is based on the observation that DM incorporated into the soil and DME in *in vitro* experiments had a direct effect on the nematode juveniles. In fact, some immobilized juveniles in DME recovered after rinsing, and DME had no effect on the nematode infectivity (number of root-invaded juveniles or root GI) in the plants.

Whatever is the mode of action of DM, this material seems to provide partial control of nematodes under natural conditions, at least in shade houses. Such partial control may gradually build up to almost complete control of the nematode in the following season if treatments with DM are repeated. Such a strategy may well fit in with nematode control in organic farming.

DM was proved in this study, as well as in our other studies (8-10), to behave as an efficient organic fertilizer. Plant growth was enhanced several-fold in treated microplots as compared with controls. Although a relatively high dose of DM was needed for nematode control, the protective and nutritional properties of DM make it an attractive alternative to chemicals, especially in an intensive organic farming system, where plant nutrition and disease control, including nematodes, are the main limiting factors.

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