

Suppression of *Meloidogyne hapla* and Its Damage to Lettuce Grown in a Mineral Soil Amended with Chitin and Biocontrol Organisms

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Abstract: Chitin was used as soil amendment in fiberglass field microplots, alone or with one or a combination of two to three species of *Hirsutella rhossiliensis*, *Paecilomyces marquandii*, *Verticillium chlamydosporium*, *Bacillus thuringiensis*, and *Streptomyces costaricanus*. Sudangrass and rapeseed were planted as cover crops and incorporated into soil as green manure amendments. Chitin amendment alone increased the marketable yield of lettuce in 1995 and reduced root-galling ratings and the reproduction of *Meloidogyne hapla* in both 1995 and 1996. Green manure amendments of sudangrass and rapeseed increased total and marketable yields of lettuce, and decreased root-galling ratings and the reproduction of *M. hapla* in 1996. *Hirsutella rhossiliensis* in combination with chitin increased total yield of lettuce over the chitin amendment alone in 1995. The combination of *B. thuringiensis*, *S. costaricanus*, and chitin either with or without *P. marquandii* increased total yield of lettuce over the chitin amendment alone in 1996. In most cases, however, the nematode-antagonistic organisms did not improve lettuce yield or further suppression of *M. hapla* compared to the chitin amendment alone. The introduced fungi were recoverable from the infested soil. The rifampicin-resistant mutant of *B. thuringiensis* was not isolated at the end of the season.

Key words: *Bacillus thuringiensis*, biological control, chitin amendment, cover crop, green manure, *Hirsutella rhossiliensis*, *Lactuca sativa*, *Meloidogyne hapla*, nematode, northern root-knot nematode, *Paecilomyces marquandii*, *Streptomyces costaricanus*, *Verticillium chlamydosporium*.

Meloidogyne hapla can be a serious problem in the commercial production of lettuce (*Lactuca sativa*) and other vegetables in New York. There is a need to develop alternatives to chemical nematicides for managing this nematode (Viaene and Abawi, 1996). Biological control options, including the use of nematode-antagonistic bacteria and fungi, soil amendment with various materials including chitin, or the incorporation of certain green manure crops, may hold potential. A cover crop of sudangrass, incorporated as a green manure, suppressed *M. hapla* and the damage it caused on lettuce in organic soil (Viaene and Abawi, 1998). *Hirsutella rhossiliensis* and *Verticillium chlamydosporium* have been reported as parasites of nematode juveniles and eggs, respectively (Bourne et al., 1994; Jaffee et al., 1991; Viaene and Abawi, in press). Recently,

soil application of *Bacillus thuringiensis*, *Paecilomyces marquandii*, and *Streptomyces costaricanus*, either in combination with chitin amendment or without soil amendment, was effective against *M. hapla* in organic soil with bulk density of 0.59 g/cm³, soil organic content of ca. 80%, and pH of 4.7 (Chen et al., in press).

The objective of this work was to examine the suppression of *M. hapla* in mineral soil amended with chitin in the presence or absence of one or a combination of the following organisms: *B. thuringiensis*, *H. rhossiliensis*, *P. marquandii*, *S. costaricanus*, and *V. chlamydosporium*. Sudangrass and rapeseed grown as cover crops and incorporated as green manure were also included for comparison.

MATERIALS AND METHODS

Preparation of biocontrol agents: *Hirsutella rhossiliensis* (IMI 265748) was provided by B. A. Jaffee. Cultures of the fungus were maintained on a half-strength potato dextrose agar (1/2 PDA) at 24 °C for 3 weeks to allow adequate sporulation. Vegetative colonies of *H. rhossiliensis* were prepared as described by Lackey et al. (1992). About

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300,000 spores of *H. rhossiliensis* were added to 500 ml PDB and incubated at room temperature on a rotary shaker at 150 rpm for 10 days. Hyphal plugs formed in the broth were homogenized in a blender for 5 seconds and centrifuged for 5 minutes. The pellets were resuspended in distilled water and mixed well into soil to a 15-cm depth at a rate of 50 ml/plant.

Verticillium chlamyosporium (CMI cc334168) was obtained from B. R. Kerry. The fungus was grown on corn meal agar (CMA) at 24 °C for 2 weeks and then transferred to 250 ml flasks containing Czapek Dox broth (CDB). The flasks were agitated on a shaking platform at room temperature for 7 days. Conidia were collected in aqueous suspension and counted in a haemocytometer. Soil was infested with *V. chlamyosporium* at a rate of 6,000 conidia/cm³ soil.

The culture and application of *B. thuringiensis* (CR371) and its rifampicin-resistant mutant (rif^r), *S. costaricanus* (CR43), and *P. marquandii* (SS-2) to soil were the same as previously reported (Chen et al., in press; Dicklow et al., 1993; Marban-Mendoza et al., 1992; Zuckerman et al., 1993). Each of them was applied as a drench and then mixed well into soil to 15-cm depth at a rate of 50 ml/plant.

Field microplots: Fiberglass field microplots (122 cm diam.) (Crosier and Abawi, 1985) containing a Lakemont silty clay loam soil (40% silt, 33% clay, 27% sand, 4.2% organic matter, pH 7.2) were established in 1995 and 1996. Chitin (Sigma, St. Louis, MO) was incorporated into the soil of appropriate microplots at a rate of 1 g/liter soil around the middle of June. Three weeks later, microplot soils were infested with *M. hapla* (ca. 4 eggs/cm³ soil), which was produced on tomato (*Lycopersicon esculentum*) cv. Rutgers grown in pasteurized soil in the greenhouse and extracted with a modified sodium hypochlorite method (Hussey and Barker, 1973). The soil of the appropriate microplots was then infested with the biocontrol organisms 3 days after infestation. Seven young seedlings of lettuce cv. Montello were transplanted into each microplot 3 to 5 days after

addition of biocontrol agents. Lettuce was harvested and the data collected 7 weeks after transplanting. Total and marketable weight of lettuce, root-galling severity, and nematode egg production were recorded at harvest. Roots were removed from microplots carefully, washed, blotted dry, and weighed. Roots were rated for root-galling severity on a scale of 1 to 9, with 1 = no galls observed, 2 = 1 to 3, 3 = 4 to 10, 4 = 11 to 25, 5 = 26 to 35, 6 = 36 to 55, 7 = 56 to 65, 8 = 66 to 80, and 9 > 80% of the roots with galls, respectively. Five grams of lettuce roots per plot were used for egg extraction by a sodium hypochlorite method (Hussey and Barker, 1973). The remaining roots were cut into small pieces and returned to the soil of the appropriate microplot.

Two sets of microplot treatments were similarly infested with *M. hapla* without the chitin amendment or the application of biocontrol organisms. One set of microplots was planted to sudangrass hybrid 'Trudan 8' (*Sorghum sudanense* × *S. sudanense*) and the other was planted to rapeseed cv. Dwarf Essex (*Brassica napus*) to determine their effects against *M. hapla*. The sudangrass and rapeseed were planted on 8 September 1995 and 11 September 1995, respectively. Plants of sudangrass and rapeseed were chopped and incorporated into soils as green manure on 17 October 1995. A total of 1.93 and 2.93 kg fresh weight of sudangrass and rapeseed, respectively, were incorporated per microplot. These quantities were equivalent to 1.64 and 1.34 metric tons dry weight/ha of sudangrass and rapeseed, respectively.

The following spring (1996), the experiment was repeated in the same microplots. Soil of each microplot was mixed thoroughly and the chitin and the biocontrol organism treatments were re-applied at the same rates as in the previous year.

Isolation of biocontrol agents: To detect *H. rhossiliensis* at harvest, a dilution plating procedure (Jaffee et al., 1991) was used. One hundred cubic centimeters of soil from the appropriate microplots were placed in a cup and incubated at 20 °C for 10 days. Three thousand second-stage juveniles of *M. hapla*

were then added. After 7 days, nematodes were extracted with a modified centrifugal-flotation method (Jenkins, 1964). Nematodes were spread on agar medium and examined under a compound microscope seven days later. The examination of a maximum of 150 *M. hapla* juveniles in each sample ceased after three individuals were observed with fungal hyphae of *H. rhossiliensis* growing out from their bodies. The infected nematodes were transferred to 1/2 PDA, and the fungal colonies developed from fungi growing out from the nematodes were compared to that of the stock culture of *H. rhossiliensis*.

Five soil samples from each plot (a total of 75) were collected to determine the survival of *P. marquandii* and *V. chlamydosporium* at harvest. A 5-g subsample was added to 100 ml of sterile water and shaken vigorously for 15 minutes. Aliquots from the prepared dilution series were added to PDA and CMA plates for detecting *P. marquandii* and *V. chlamydosporium*, respectively. Each plate was swirled to distribute the soil suspension and was incubated at 22 °C in the dark (Kerry et al., 1993; Marban-Mendoza et al., 1992). Characteristic colonies were transferred to the appropriate agar medium for additional examination and confirmation.

The survival of *B. thuringiensis* in mineral soil at harvest was determined with the same procedure as described previously (Chen et al., in press).

Analysis: A randomized complete block design was used with five replicates for each treatment. Plant weights, root-galling severity, and reproduction data were subjected to ANOVA. The Least Significant Difference (LSD) test was performed to separate means. Contrasts were performed with MSTAT-C (Department of Crop and Soil Science, Michigan State University, East Lansing, MI). The reproduction factor (Rf) of *M. hapla* for the first season was calculated as $Rf = Pf_{95}/Pi_{95}$, where Pf_{95} was the final egg population density of *M. hapla* (Pf) in 1995 and Pi_{95} was the initial egg population density added to soil in 1995. For the second season Pf was calculated as $Rf = Pf_{96}/Pf_{95}$,

where Pf_{95} was considered as the Pi of *M. hapla* to include cover crop-green manure treatments.

RESULTS

Soil infestation with *M. hapla* at 4 eggs/cm³ soil resulted in severe infection and high reproduction of the nematode on roots of lettuce, causing significant reduction in total and marketable weight of lettuce in 1995. Root galling was severe, and total and marketable weight was suppressed by *M. hapla* in 1996 (Tables 1, 2).

The chitin amendment alone reduced root-galling severity and lowered the reproduction of *M. hapla* on lettuce roots ($P \leq 0.05$). Amending the soil with chitin increased total yield of lettuce compared to that of lettuce in the control plots in both 1995 and 1996, but marketable yield of lettuce was significantly increased only in 1995 (Table 1).

The sudangrass and rapeseed cover crops, incorporated as green manure, also increased ($P \leq 0.05$) total and marketable yields of lettuce, reduced root-galling severity, and lowered the reproduction of *M. hapla* in 1996 (Tables 1, 2). In addition, the green manure amendments of sudangrass and rapeseed reduced root-galling severity and lowered egg production of *M. hapla* ($P \leq 0.05$) more than did the chitin amendment with and without biocontrol organisms (Table 2).

The reproduction factor (Rf) for *M. hapla* in the non-amended soils was 1.9–2.4 and 0.4 in 1995 and 1996, respectively. Soil amendment with chitin reduced Rf of *M. hapla* in both 1995 and 1996 (Table 1). Similarly, green manures of sudangrass and rapeseed reduced Rf in 1996.

The effect of the various biocontrol organisms on yield of lettuce, root-galling severity, and reproduction of *M. hapla* was inconclusive and generally did not differ significantly from the chitin amendment alone (Tables 1, 2). However, a few treatments containing one or a combination of biocontrol organisms with the chitin amendment increased lettuce yield or reduced nematode damage and reproduction (Tables 1,

TABLE 1. Influence of chitin, biocontrol organisms, and cover crops on *Meloidogyne hapla* and its damage to lettuce in field microplots.^a

	Total yield (g)/plant		Marketable yield (g)/plant		Root-galling severity ^c		Reproduction factor ^d	
	1995	1996	1995	1996	1995	1996	1995	1996
Nematode, amendment ^b								
Mh	638 cd	486 d	428 de	347 c	6.9 a	7.0 a	2.05	0.42
Mh, sudangrass	— ^e	582 abc	—	431 ab	—	5.7 b	—	0.07
Mh, rapeseed	—	565 abc	—	423 ab	—	5.4 b	—	0.15
Mh, chitin	703 bc	512 bcd	499 bc	401 bc	5.0 c	4.2 c	0.72	0.17
Mh, chitin/Hr	778 a	512 bcd	559 ab	406 bc	5.0 c	3.7 c	0.63	0.16
Mh, chitin/Hr/Pm	688 c	505 cd	480 cd	393 bc	5.3 c	3.7 c	0.79	0.12
Mh, chitin/Hr/Vc	763 ab	585 ab	541 abc	435 ab	5.1 c	3.9 c	0.67	0.15
Mh, chitin/Bt/Sc	680 c	613 a	478 c	461 ab	6.1 b	4.1 c	1.28	0.08
Mh, chitin/Bt/Sc/Pm	607 d	632 a	409 e	498 a	7.1 a	3.9 c	1.52	0.10
Control	816 a	589 a	602 a	421 ab	1.0 d	1.0 d	0.00	0.00

^a The plots designed for cover crops were planted with lettuce and inoculated with *M. hapla*, without addition of chitin and biocontrol agents. After harvesting lettuce, sudangrass and rapeseed were planted on 8 September 1995 and 11 September 1995, respectively, and incorporated into soil on 17 October 1995. Means in a column followed by the same letter are not significantly different according to an LSD test ($P = 0.05$).

^b Control: without addition of *M. hapla*, chitin, and biocontrol agents; Bt: *Bacillus thuringiensis*; Hr: *Hirsutella rhossiliensis*; Mh: *M. hapla*; Pm: *Paecilomyces marquandii*; Sc: *Streptomyces costaricanus*; Vc: *Verticillium chlamydosporium*.

^c Rating scale for galling: 1: no galls observed; 2: 1 to 3%; 3: 4 to 10%; 4: 11 to 25%; 5: 26 to 35%; 6: 36 to 55%; 7: 56 to 65%; 8: 66 to 80%; 9: >80% of the roots with galls.

^d Reproduction factor (Rf): Pf/Pi, where Pi and Pf were the initial and final egg population density of *M. hapla*, respectively.

^e Not included in 1995.

2). *Hirsutella rhossiliensis* in combination with chitin increased total lettuce yield over the chitin amendment alone in 1995 ($P \leq 0.05$). *Bacillus thuringiensis* and *S. costaricanus* in combination with chitin in the presence or absence of *P. marquandii* increased total lettuce yield over the chitin amendment in 1996 ($P \leq 0.05$) (Tables 1, 2). The treatment of chitin plus bacteria also increased marketable yield, compared with the chitin amendment alone in 1996 (Table 2).

In this investigation, *H. rhossiliensis* was isolated from soil at end of season in both

years where it was applied alone (Table 3). However, it was detected only in one of the two years when added in combinations with another biocontrol organism. *Paecilomyces marquandii* was isolated when applied with *H. rhossiliensis* in both years, but it was not detected in 1996 from soils also infested with the bacteria. *Verticillium chlamydosporium* applied with *H. rhossiliensis* was detected in 1995, but not in 1996. The rifampicin-resistant mutant of *B. thuringiensis* was not isolated from the *M. hapla*-infested soil at the end of the 1996 growing season (Table 3).

TABLE 2. Contrasts for the biocontrol effects on *Meloidogyne hapla* in field microplots.

Contrasts ^a	Total yield		Marketable yield		Root galling		Egg production	
	1995	1996	1995	1996	1995	1996	1995	1996
Amendments vs. <i>M. hapla</i> alone	+* ^b	+*	+*	+*	-*	-*	-*	-*
BCA + chitin vs. <i>M. hapla</i> alone	+*	+*	+*	+*	-*	-*	-*	-*
BCA + chitin vs. chitin alone	n.s.	+*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fungi + chitin vs. chitin alone	n.s.	+*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Bacteria + chitin vs. chitin alone	-*	+*	-*	+*	+*	n.s.	n.s.	n.s.
Cover crops vs. <i>M. hapla</i> alone	ND	+*	ND	+*	ND	-*	ND	-*
Cover crops vs. BCA + chitin	ND	n.s.	ND	n.s.	ND	+*	ND	+*

^a Cover crops (sudangrass and rapeseed) were planted on 8 September 1995 and 11 September 1995, respectively, and incorporated into soil on 17 October 1995. BCA: biological control agents. ND: not included in 1995.

^b Increase (+) or reduction (-) of lettuce yield, root-galling severity, and egg production followed by "*" indicates a significant difference ($P \leq 0.05$); n.s. indicates $P > 0.05$.

TABLE 3. Detection of biocontrol organisms applied alone or in combination in field microplots.

Organism	1995	1996	Application
<i>Hirsutella rhossiliensis</i> (Hr)	+ ^a	+	Alone
	+	-	Combination with Pm
	-	+	Combination with Vc
<i>Paecilomyces marquandii</i> (Pm)	+	+	Combination with Hr
	+	-	Combination with Bt and <i>Streptomyces costaricanus</i> (Sc)
	+	-	Combination with Hr
<i>Verticillium chlamydosporium</i> (Vc)	+	-	Combination with Hr
<i>Bacillus thuringiensis</i> (Bt)	ND ^b	-	Combination with Sc
	ND	-	Combination with Sc and Pm

^a Organism was (+) or was not (-) detected in soil at end of season.

^b ND: not investigated in 1995. The survival of *Streptomyces costaricanus* was not investigated in 1995 and 1996.

DISCUSSION

Meloidogyne hapla caused severe damage to lettuce grown on silty clay loam soils under field microplot conditions. This nematode also has been documented to cause severe damage to lettuce grown on organic soil (Chen et al., 1996; Viaene and Abawi, 1996, 1998). In a previous study, *B. thuringiensis*, *S. costaricanus*, *P. marquandii*, and soil amendments of wheat mash, chitin, or brewery compost lowered root-galling severity, reduced reproduction of *M. hapla*, and increased lettuce yield in organic soil. However, there was no interaction between biocontrol organism and soil amendment (Chen et al., in press). In this investigation, the addition of nematode-antagonistic organisms to the chitin-amended mineral soil generally did not suppress *M. hapla* or increase lettuce yield, compared with the chitin treatment alone.

Several other studies have reported that combining biocontrol organisms and chitin amendments did not result in synergistic or additive effects. Viability of *P. lilacinus* was low in chitin-amended soil stored at 25 °C, compared to its survival on wheat, granules, and pellets (Cabanillas et al., 1989). The treatment of seeds with two antagonists and chitin addition to soil incorporated with antagonists did not have significant effects on the control of cucumber wilt (Cho et al., 1989). Actinomycetes, chitinase-producing microorganisms, and chitinase activity were markedly stimulated by addition of chitin in soil, whereas the population of bacteria was

not altered appreciably, and certain soil fungi were suppressed (Mitchell and Alexander, 1962). It was suggested that mycolytic activity and toxin production might be implicated in the selective influence of chitin amendment in suppressing specific detrimental and beneficial species.

In this study, the chitin amendment alone significantly increased the marketable yield of lettuce in 1995 and reduced root-galling severity and reproduction of *M. hapla* in both years. Similar results have also been observed in other studies (Rodríguez-Kábana et al., 1984; Segers and Coosemans, 1990; Spiegel et al., 1986, 1987, 1988, 1989). ClandoSan, a chitin-urea soil amendment, for example, was registered by the U.S. Environmental Protection Agency as a biological nematicide for use as a pre- and postplant soil treatment (Westerdahl et al., 1992). Chitin is present in gelatinous matrix and egg shells of *Meloidogyne* and other nematodes (Spiegel et al., 1987). It would seem that the action of chitin is associated either with the microbiological production of antibiotics or of substances capable of degrading chitin in nematode egg walls.

Under our research conditions, the incorporation of green manures of sudangrass and rapeseed were effective in suppressing the population of *M. hapla* and increasing lettuce weight. Cover crops of sudangrass and rapeseed incorporated as green manures were effective against *M. chitwoodi* on potato (Mojtahedi et al., 1991, 1993a, 1993b). Recently, Viaene and Abawi (1998) reported that sudangrass 'Trudan 8' was ef-

fective in reducing populations of *M. hapla* and its damage to lettuce in organic soils in New York. The addition of organic materials to soil stimulates activity of actinomycetes, algae, bacteria, fungi, microbivorous nematodes, and others (Rodríguez-Kábana et al., 1987; Sayre, 1980). The deleterious effect on plant-parasitic nematodes can be associated with the increased populations of parasitic or antagonistic organisms as well as the accumulations of decomposition products and microbial metabolites (Badra et al., 1979; Godoy et al., 1983; Kahn et al., 1974; Walker, 1971).

The survival of introduced organisms in field soils is important for the success of biological control. *Bacillus thuringiensis* was unable to remain viable more than 49 days in organic soil. The population density of the bacterium is negatively correlated to the length of time it resides in soil (Chen et al., in press). In this investigation, the survival of the same rif^r strain of *B. thuringiensis* also was not detectable in mineral soil after 7 weeks. The survival of introduced *Pseudomonas fluorescens*, especially the modified strain, was influenced by soil texture (Guimaraes et al., 1998). The persistence of the nematophagous fungi in mineral soil might be influenced by the competitiveness among the fungi introduced and also the population level of *M. hapla* under the prevailing research conditions. *Hirsutiella rhossiliensis* was dependent on the presence of host nematodes for survival (Jaffee et al., 1992). Further research, including a more quantitative approach, is needed to explore the antagonism among biocontrol agents and between introduced organisms and certain soil amendments.

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