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Efficacy of *Bacillus thuringiensis, Paecilomyces marquandii,* and *Streptomyces costaricanus* with and without Organic Amendments against *Meloidogyne hapla* Infecting Lettuce

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Abstract: Chitin, wheat mash, or brewery compost were incorporated into unfumigated and methyl bromide-fumigated organic soils placed in microplots formed from cylindrical drainage tiles (0.25 m-diam. clay tile). After 3 weeks, *Meloidogyne hapla* and cell or spore suspensions of *Bacillus thuringiensis, Paecilomyces marquandii,* and *Streptomyces costaricanus* were individually added to the soils of designated microplots. A *B. thuringiensis* + *S. costaricanus* combination was also tested. Lettuce seedlings, cv. Montello, were transplanted into the soils 3 to 4 days later. All the bacterial and fungal antagonists applied without a soil amendment, except the *B. thuringiensis* + *S. costaricanus* treatment, reduced root galling and increased lettuce head weight in the unfumigated organic soil, but not in the fumigated soil. All three amendments were also effective against *M. hapla* and reduced root galling in fumigated and unfumigated soils. Wheat mash amendment increased lettuce head weight in the unfumigated soil. In general, no antagonist × amendment interaction was detected. Soil populations of *B. thuringiensis* were maintained at $\geq$4.0 $\log_{10}$ colony-forming units/g organic soil during the first 14 days after planting. However, viable cells of *B. thuringiensis* were not detected after 49 days.

Key words: Bacterial antagonists, biological control, brewery compost, chitin, field microplots, fungal antagonists, *Lactuca sativa* L., *Meloidogyne hapla,* nematode, northern root-knot nematode, soil amendments, wheat mash.

The northern root-knot nematode, *Meloidogyne hapla* Chitwood, is a major pathogen that causes significant losses in lettuce (*Lactuca sativa* L.) and other vegetable crops grown on organic soils in New York (Viaene and Abawi, 1996). Severely infected lettuce plants often fail to produce marketable heads and, thus, are often not harvested. Commercially acceptable lettuce cultivars grown in New York are susceptible to this nematode (Abawi and Robinson, 1991). Crop rotation is of limited value for control of *M. hapla* in organic soil in New York because all crops rotated with lettuce (e.g., onion, carrot, potato, bean) are susceptible to this nematode. At present, preplant soil fumigation with toxic and expensive nematicides is the only practical option available to lettuce growers for the control of this nematode on organic soils. However, growers have experienced variable results with the efficacy of this soil fumigation, possibly due to the method of application, soil conditions, and environmental factors. Thus, there is a need to develop alternative strategies and tactics, including biological control, to manage this nematode.

Considerable information available in the literature has documented the effectiveness of several biological control agents to manage plant-parasitic nematodes (Chilcott and Wigley, 1994; Jaffee et al., 1991; Kerry et al., 1993; Meyer and Meyer, 1996; Siddiqui and Mahmood, 1997; Walia, 1996). Recently, isolates of *Bacillus thuringiensis* and *Streptomyces costaricanus* were reported to be effective for reducing populations of *Caenorhabditis elegans* in laboratory tests and *M. incognita* and *Pratylenchus penetrans* in greenhouse tests (Dicklow et al., 1993; Zuckerman et al., 1993). *Paecilomyces marquandii* was considered one of the natural soil organisms that contributed to nematode suppression in chinampa agricultural soils in Mexico (Marban-Mendoza et al., 1992). However, there was no information on the effects of these organisms against *M. hapla* infecting lettuce.

The incorporation of soil amendments

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also may be effective in controlling nematodes, primarily by altering the soil microflora (Godoy et al., 1983; Rodríguez-Kábana et al., 1987). One possible change is an increase in nematophagous fungal species (Esnard et al., 1998). In addition, a nematicidal effect may be associated with breakdown of nitrogenous materials (Sikora, 1992). The addition of large amounts of chitin amendments to soil was shown to greatly enhance chitinase and other enzymatic activities due to stimulated chitin-decomposing microflora (Culbreath et al., 1985). Because chitin is a component of the eggshell of nematodes, chitinase-producing microorganisms also are effective in destroying nematode eggs. Other organic amendments may also be effective. Yield of squash was increased by 155% with the incorporation of compost as compared to the unamended control (McSorley and Gallaher, 1995). In a greenhouse study, egg mass production of *M. javanica* and *M. incognita* was significantly reduced by amending soils with ground wheat seed at rates of 1.0 and 2.0% (Rich and Rahi, 1995). Three greenhouse tomato trials at Amherst, Massachusetts, indicated that the incorporation of wheat mash reduced root galling incited by *M. incognita* by ca. 50 to 80% (Zuckerman, unpubl. data). Combining the use of chitin or other amendments with promising nematophagous organisms might result in enhanced biological control activities against plant-parasitic nematodes.

The objective of this study was to determine the efficacy of *B. thuringiensis, P. marquandii,* and *S. costaricanus,* with and without an amendment of chitin, brewery compost, or wheat mash, against *M. hapla* and its damage to lettuce grown in organic soil in field microplots.

Materials and Methods

Field microplots: Clay tile microplots (made with cylindrical unglazed drainage tiles, 25-cm-internal diam., 30-cm-long) were filled with either unfumigated or methyl bromide-fumigated organic soil obtained from a commercial lettuce field near Oswego, New York. The organic soil was a Carlisle muck soil with a bulk density of 0.59 g/cm^3, a soil organic content of ca. 80%, and a pH of 4.7. Brewery compost (10 g/liter soil, Nutri Brew, Baldwinsville, NY), chitin (1 g/liter soil, Sigma, St. Louis, MO), or wheat mash (10 g/liter soil, ground-up wheat and barley in equal amounts) were incorporated into designated microplot soils to a depth of 15 cm. The plots were watered and maintained for 3 weeks to give organic materials time to decompose and allow phytotoxic materials to disperse (Westerdahl et al., 1992) and were then infested with *M. hapla* eggs (20 eggs/cm^3 soil) mixed with soil to a depth of 15 cm. Inoculum suspensions of *B. thuringiensis, P. marquandii, S. costaricanus,* or *B. thuringiensis* + *S. costaricanus* were applied as drenches and then mixed well into soil to a depth of 15 cm at a rate of 50 ml/plant. Four days later, two lettuce seedlings were transplanted into each of the microplots. To obtain the seedlings, seeds of lettuce cv. Montello were planted in pasteurized soil and maintained in the greenhouse for 2 weeks. The seedlings were hardened for 1 week in cold frames before being transplanted into the microplots. Lettuce was harvested 7 weeks after transplanting. All treatments in fumigated or unfumigated organic soils were randomized with five replicates. Treatments included main effects of antagonists (Fig. 1) and amendments (Fig. 2). Some of the combination treatments are listed in Table 1. The experiment was conducted in 1995 and repeated in 1996. Total and marketable weight of lettuce, root galling, and egg production were recorded at harvest.

Antagonistic organisms: Bacillus thuringiensis was stored at −80 °C in cryopreservation buffer consisting of 100 ml 1-M NaCl, 50 ml 1-M phosphate buffer, 300 ml glycerol, and 3.0 ml 0.1-M $MgSO_4$/liter. A rifampicin-resistant mutant (rif$^+$) of the wild-type *B. thuringiensis* (CR371) was selected on a solid medium amended with rifampicin by the method of Liu and Sinclair (1992). *Bacillus thuringiensis* (0.1 ml cryopreserved cell suspension) was grown in nutrient broth on a rotary shaker for 6 days at 28 °C. The cells were pelleted by centrifugation at 10,410*g* for 10 minutes. The pellet was resuspended

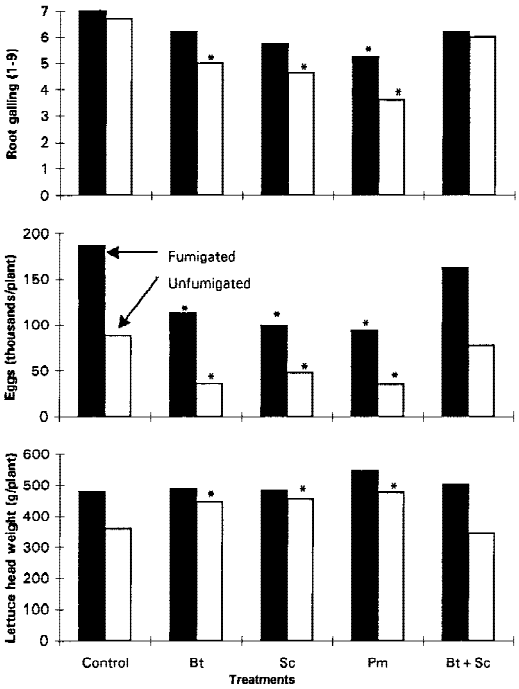

FIG. 1. Effects of *Bacillus thuringiensis* (Bt), *Streptomyces costaricanus* (Sc), and *Paecilomyces marquandii* (Pm) on lettuce head weight, and on root galling and reproduction of *Meloidogyne hapla* on lettuce, without addition of amendments. Root galling was rated on a scale of 1 (no visible symptoms) to 9 (>80% of the roots galled). Bar with an asterisk indicates there is a significant difference between the treatment and the corresponding control according to Student's *t*-test ($P = 0.05$).

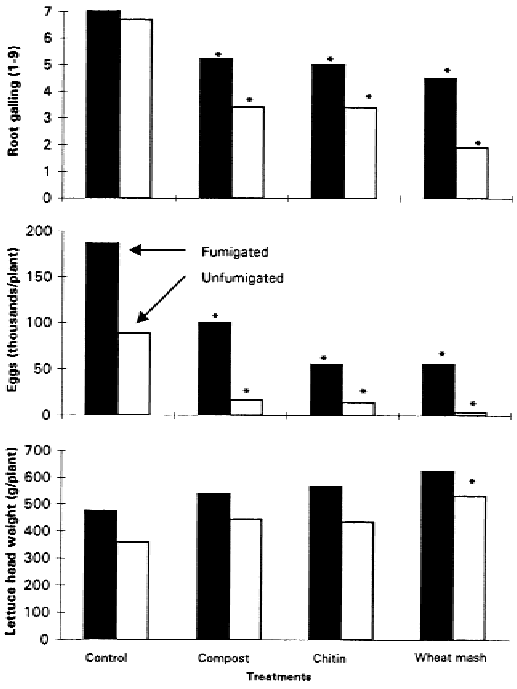

FIG. 2. Effects of brewery compost, chitin, and wheat mash amendments in soils on lettuce head weight, and on root galling and reproduction of *Meloidogyne hapla* on lettuce. Root galling was rated on a scale of 1 (no visible symptoms) to 9 (>80% of the roots galled). Bar with an asterisk indicates there is a significant difference between the treatment and the corresponding control according to Student's *t*-test ($P = 0.05$).

in 100 ml of distilled water, and the final concentration of the inoculum suspension was 1×10^7 colony-forming units (CFU)/ml. Each microplot received 100 ml of the bacterial suspension, which was mixed well into the soil to a 15-cm depth.

Inoculum of *Streptomyces costaricanus* (CR43) was prepared by incubating 1 ml of a cryopreserved cell suspension in 100-ml batches of potato dextrose broth (PDB) for 5 days with vigorous shaking on an orbital shaker at room temperature. The inoculum was concentrated by centrifugation at 10,410*g* for 10 minutes. The pellet was resuspended in 100 ml of distilled water, which provided ca. 1×10^5 CFU/ml. The prepared suspension of *S. costaricanus* was applied as a drench and then mixed well into soil at a rate of 50 ml/plant.

An isolate of *P. marquandii,* SS-2, which was originally obtained from nematode-suppressive chinampa soils near Mexico City (Marban-Mendoza et al., 1992), was maintained on PDA at 25 °C in the dark. The inoculum was prepared by transferring a 15-mm plug of a 1-wk-old culture of the fungus into PDB and incubating the culture on a shaker at 125 rpm for 10 days at room temperature. The liquid culture was centrifuged at 10,410*g* for 10 minutes, and the inoculum pellet was resuspended in distilled water and adjusted to the original volume. The fungal inoculum suspension also was applied as a drench (as described above), and mixed well into soil.

Survival of B. thuringiensis in soil: Survival of *B. thuringiensis* in soil was determined by following changes in populations of the rif^+ mutant strain in 1996. Approximately 50 g of rhizosphere soils was collected from the

TABLE 1. Effects of soil amendments and microbial antagonists on lettuce yield and on root galling and reproduction of *Meloidogyne hapla*.

Treatment[a]	Head weight (grams per plant)	Root-galling rating[b]	Eggs per gram of root
Fumigated soil (F)	82.5 ab[c]	7.8 a	35,853 a
Unfumigated soil (NF)	80.6 b	7.2 a	23,857 ab
F + wheat mash	106.3 a	4.0 c	4,952 c
NF + wheat mash	83.5 ab	4.0 c	5,548 c
F + compost	93.5 ab	7.0 a	33,204 a
NF + compost	99.4 ab	7.0 a	21,582 ab
F + wheat mash + compost	96.6 ab	4.0 c	9,097 bc
NF + wheat mash + compost	100.9 ab	4.0 c	5,866 c
F + wheat mash + Bt[d]	81.6 b	5.8 b	18,708 b
NF + wheat mash + Sc[e]	98.2 ab	3.6 c	3,854 c
NF + wheat mash + Pm[f]	100.5 ab	4.0 c	2,080 c

[a] All treatments were infected initially with *M. hapla* eggs and treated with antagonistic organisms and soil amendments or untreated.
[b] Root galling was scored on a scale of 1 (no visible symptoms) to 9 (>80% of the roots with galls).
[c] Means within a column with a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.
[d] Bt = *Bacillus thuringiensis*.
[e] Sc = *Streptomyces costaricanus*.
[f] Pm = *Paecilomyces marquandii*.

appropriate treatments at 1-week intervals for the first 3 weeks after application and at harvest. The weekly precipitation during the 3 weeks following the application of *B. thuringiensis* was 44, 32, and 16 mm. A 5-g subsample of each sample was vigorously agitated in 20 ml of water on a shaker for 20 minutes. Five ml of phosphate-glycerol buffer was added immediately to reduce the incidence of cell-burst (Esnard et al., 1994). The soil suspension was then passed through a series of sieves: 100-mesh (150-µm), 325-mesh (44-µm), and 500-mesh (25-µm). The resulting suspensions were centrifuged at 810*g* for 3 minutes, and a dilution series of each was prepared. One hundred microliters of each soil dilution was then spread on nutrient agar plates amended with 100 µg/ml rifampicin (NA^{rif}). Plates were incubated at 28 °C for 48 hours and the total number of CFU recorded under a dissecting microscope at ×10. Counts were represented as $\log_{10}$ number of bacteria per gram of soil. The *B. thuringiensis* population (Y) dynamics over time (X) were described in linear and second-degree polynomial models.

Assessment: Root galling and reproduction of *M. hapla* were determined at harvest. Plant roots were washed, weighed, and rated for root galling on a scale from 1 (no visible symptoms) to 9 (>80% of the roots with galls). Ratings of 2 to 8 indicated 1 to 3, 4 to 10, 11 to 25, 26 to 35, 36 to 55, 56 to 65, and 66 to 80% of the roots with galls, respectively. Reproduction of *M. hapla* per root system (eggs per plant) was determined by extracting the eggs from the root systems with a sodium hypochlorite method (Hussey and Barker, 1973).

Fresh lettuce head and root weights in each treatment were recorded. The combined 2-year data were used for factorial and correlation analyses. The results in the second year (1996), when *B. thuringiensis* population dynamics in the organic soil were also investigated, were used for mean separation. Student's *t*-test was conducted to detect if there was a significant ($P \leq 0.05$) difference between the individual treatments and the control. The $\log_{10}$ number of CFU per gram of soil of *B. thuringiensis* was used for assessing its survival in soil.

A greenhouse post-season bioassay was conducted after the harvest in 1995 to assess the residual effects of the antagonists and amendments in soil samples collected from selected treatments established in the field microplot test. Approximately 1,000 cm^3 of soil was dug from each microplot on 24 Oc-

tober 1995. About 500 cm^3 of each sample was placed in 10-cm clay pots that were arranged in a randomized block design with five replicates. Lettuce seedlings were transplanted on 31 October 1995 and harvested 8 weeks later. Root galling and reproduction of *M. hapla* were determined at harvest. Fresh lettuce head weight was recorded.

Results

Fresh head weight of lettuce grown in the fumigated soil was greater than that of lettuce grown in the unfumigated soil in all the treatments (Figs. 1,2). The addition of *B. thuringiensis, S. costaricanus,* or *P. marquandii* significantly reduced egg production of *M. hapla* in both the fumigated and unfumigated soils (Fig. 1). In the fumigated soil without a soil amendment, the addition of *B. thuringiensis, S. costaricanus,* and *P. marquandii* increased head weight of lettuce by 2.1%, 1.3% and 14.3%, respectively; decreased root galling by 11.4%, 18.6% and 25.7%, respectively; and reduced egg production of *M. hapla* by 25.4%, 31.3%, and 46.3%, respectively. In the unfumigated soil without a soil amendment, the *B. thuringiensis, S. costaricanus,* and *P. marquandii* treatments significantly increased lettuce head weight and reduced root galling. However, the combined *B. thuringiensis* + *S. costaricanus* treatment failed to increase lettuce head weight or reduce egg production and root galling by *M. hapla* in both fumigated and unfumigated soils (Fig. 1).

Application of the brewery compost, chitin, or wheat mash alone significantly reduced root galling and egg production of *M. hapla* in both the fumigated and unfumigated soils ($P \leq 0.05$) (Fig. 2). The addition of brewery compost, chitin, and wheat mash amendments increased head weight of lettuce by 13.4%, 18.9%, and 31.1%, respectively, in the fumigated soil and by 23.3%, 20.6%, and 47.8%, respectively, in the unfumigated soil (Fig. 2). Incorporation of wheat mash at 10 g/liter soil reduced the root galling from 6.7 to 1.9 (on a scale of 1 to 9) and increased lettuce head weight from 360 to 532 g/plant in the unfumigated soil ($P \leq 0.05$).

Factorial analysis of the data indicated that the soil amendment factor was statistically significant ($P \leq 0.05$) under the experimental conditions. The antagonist factor was also significant, except for the head weight of lettuce in both the fumigated ($P = 0.195$) and unfumigated ($P = 0.058$) soils. No antagonist × amendment interaction was detected, except for root galling in the unfumigated soil. In general, combination treatments were not more effective than the antagonists or amendments alone. The coefficient of variation indicated that the precision of the experiment ranged from 2.4% to 16.6%.

The relationship between root galling and reproduction of *M. hapla* is shown in Figure 3. As root-galling rating rose, egg production of *M. hapla* increased. The root-galling rating on the average was 5.8 in the fumigated soil and 4.4 in the unfumigated soil. On the average, ca. 117,000 eggs per plant were discovered in the fumigated soil, com-

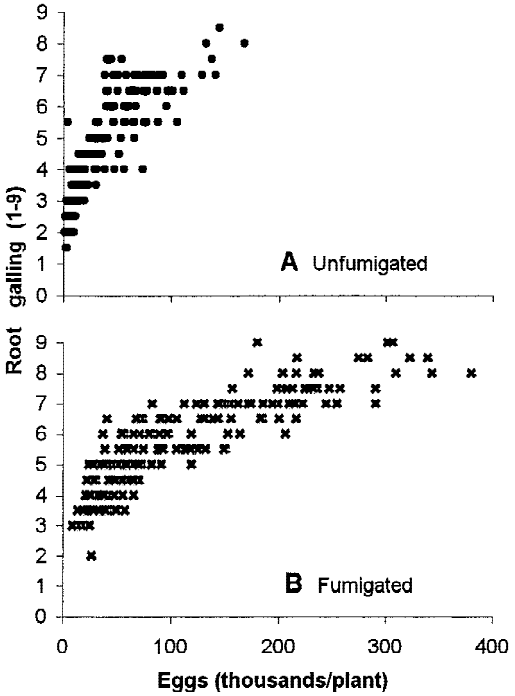

Fig. 3. Relationship of root galling and reproduction of *Meloidogyne hapla* at the end of the growing season. A) Unfumigated plots. B) Fumigated plots. Root galling was rated on a scale of 1 (no visible symptoms) to 9 (>80% of the roots galled).

pared to ca. 36,000 eggs per plant in the unfumigated soil. High root-galling ratings were associated with fewer eggs in the unfumigated soil than in the fumigated soil.

In a post-harvest greenhouse bioassay, root-galling was lower in soils collected from most of the tested treatments of antagonists and organic amendments established in the microplot test ($P \leq 0.05$) compared to untreated, fumigated, and unfumigated controls. Only compost treatment failed to reduce root galling (Table 1). Egg production of *M. hapla* in all treatments except compost also was lower than in the corresponding controls. The results obtained suggested that, except for compost treatments, the selected antagonists and organic amendments continued to exert a similar effect against *M. hapla* as that observed in the field. However, lettuce head weights were not affected by the treatments.

In general, population densities of *B. thuringiensis* in unfumigated soils were lower with wheat mash amendment than in soils without amendment (Table 2). In some cases, population densities of *B. thuringiensis* in fumigated soils were lower with chitin amendment than in soils without amendment (Table 2). Populations of *B. thuringiensis* on the average were 5.3, 4.4, 4.3, and 3.0 $\log_{10}$ CFU/g soil at 0, 7, 14, and 21 days after planting, respectively, and were described in linear and second-degree polynomial models (Fig. 4). However, viable cells of *B. thuringiensis* were not detected from soil samples collected at harvest, 49 days after its application at planting, and were not predicted by either the linear or the second-degree polynomial models.

Discussion

In this investigation, *M. hapla* caused severe damage to lettuce grown on organic soil under field microplot conditions. *Bacillus thuringiensis, S. costaricanus,* and *P. marquandii* were effective in lowering root galling, reducing reproduction of *M. hapla,* and increasing lettuce head weight in the unfumigated organic soil. The biocontrol organisms also reduced reproduction of *M. hapla* in the fumigated soil.

Biological control agents of soilborne plant pathogens often are applied to soils in combination with organic materials (Mittal et al., 1995; Rodríguez-Kábana et al., 1987).

Table 2. Survival of *Bacillus thuringiensis* (Bt) in fumigated or unfumigated organic soils infested with *Meloidogyne hapla,* without or with an amendment (wheat mash [WM] or chitin), and without or with *Streptomyces costaricanus* (Sc), in field microplots planted to lettuce in 1996.

	Population of *B. thuringiensis* ($\log_{10}$ CFU/g soil)[a]				
Treatment	18 June	25 June	2 July	10 July	20 August
Fumigated					
Bt	5.44 ab[b]	4.60 a	4.52 ab	3.08 ab	0
Bt + WM[c]	5.11 b	4.22 ab	4.34 b	2.90 b	0
Bt + Chitin	5.00 bc	3.82 b	3.88 c	2.78 b	0
Bt + Sc	5.38 ab	4.34 ab	4.22 b	2.78 b	0
Bt + Sc + WM	4.90 c	4.00 b	3.92 c	2.60 b	0
Bt + Sc + chitin	5.27 b	4.56 a	4.45 ab	3.20 ab	0
Unfumigated					
Bt	5.79 a	4.62 a	4.70 a	3.38 a	0
Bt + WM	5.08 bc	4.40 ab	4.20 bc	2.90 b	0
Bt + Chitin	5.31 ab	4.74 a	4.71 a	3.51 a	0
Bt + Sc	5.77 a	4.22 ab	4.30 b	3.20 ab	0
Bt + Sc + WM	5.19 b	4.22 ab	4.15 bc	2.78 b	0
Bt + Sc + chitin	5.16 b	4.52 a	4.48 ab	3.20 ab	0

[a] CFU = Colony-forming units.
[b] Means within a column with a common letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test.
[c] Wheat mash consisted of ground-up wheat and barley in equal proportion.

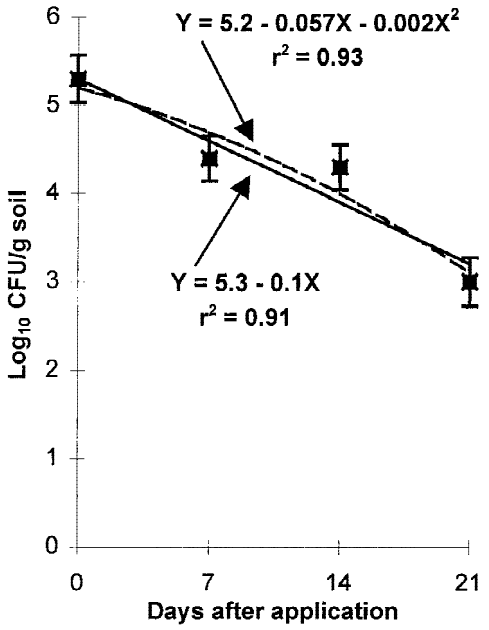

FIG. 4. Population dynamics of rifampicin-resistant mutant of wild-type *Bacillus thuringiensis* (Bt) CR-371 under field microplot conditions in 1996. The bacterial populations are represented as $\log_{10}$ colony-forming units(CFU)/g of soil. Squares are the observed Bt populations. Solid line is the predicted Bt population with a linear function. Dotted line is the predicted Bt population with a polynomial function. Bars indicate standard deviation. No colonies of Bt from soil samples were obtained on Day 49, the end of the season.

Organic materials contribute to enhanced biological activities against the target pathogen by providing the needed nutrients for initial growth of the biocontrol agents in soil, and may be used as carriers to facilitate distribution. Breakdown of organic materials may release toxic and nematicidal substances that contribute to nematode control. Additionally, organic residues have been associated with a buildup of free-living nematodes in soils where nematophagous fungi were present, and high infection of free-living nematodes contributed to the control of plant-parasitic nematodes (Esnard et al., 1998). However, in the current study, the efficacy of biocontrol organisms against *M. hapla* was not significantly increased by addition of soil amendments in the organic soil. Similar observations have been reported previously. Organic amendments did not enhance parasitism of nematodes by *Hirsutella rhossiliensis* (Jaffee et al., 1994). *Paecilomyces lilacinus* was commonly isolated from chitin-amended soil, but the addition of chitin did not increase egg parasitism (Stirling and West, 1991).

In this investigation, wheat mash, chitin, and brewery compost were effective in field microplot tests against *M. hapla,* reducing root-galling severity on lettuce in organic soil. Wheat mash also increased lettuce head weight. The potential of various organic amendments including chitin, brewery compost, and wheat mash to control plant-parasitic nematodes has been recognized and reported in the literature (Craft and Nelson, 1996; Culbreath et al., 1985; Nelson and Craft, 1992; Rodríguez-Kábana, 1986). Greenhouse tests at the University of Massachusetts indicated that wheat mash at low rates (0.5–1.0%, w/v) in mineral soil was effective against *M. incognita* on tomato (B. M. Zuckerman, unpubl. data). Certain amendments may contribute to the establishment of a healthier rhizosphere environment for the growth of lettuce plants; for example, wheat mash amendment increased the total number of soil nematodes, including *Aphelenchus* sp. (Chen et al., 1996). The suppression of *M. hapla* observed in these trials may be the result of an increase in soil microbial activities, which warrants further investigation to ascertain the mechanisms involved.

Rifampicin markers have been used to facilitate investigation of root colonization capacity and survival of biocontrol organisms in soil (Press et al., 1992; Sebald, 1993). In this investigation, the population density of a rif^+ mutant of *B. thuringiensis* declined over time, and the bacterium was not detected from soil samples collected at harvest. Population levels of the bacterium in soil were influenced by rainfall and organic amendments. The use of rifampicin markers appears to be a useful technique in assessing the population dynamics of bacterial biocontrol agents.

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