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Hirsutella rhossiliensis and *Verticillium chlamydosporium* as Biocontrol Agents of the Root-knot Nematode *Meloidogyne hapla* on Lettuce

Nicole M. Viaene and George S. Abawi[1]

Abstract: *Hirsutella rhossiliensis* and *Verticillium chlamydosporium* infected second-stage juveniles (J2) and eggs of *Meloidogyne hapla,* respectively, in petri dishes and in organic soil in pots planted to lettuce in the greenhouse. In vitro, *H. rhossiliensis* produced 78 to 124 spores/infected J2 of *M. hapla.* The number of J2 in roots of lettuce seedlings decreased exponentially with increasing numbers of vegetative colonies of *H. rhossiliensis* in the soil. At an infestation of 8 *M. hapla* eggs/cm^3 soil, 1.9 colonies of *H. rhossiliensis*/cm^3 soil were needed for a 50% decrease in J2 penetration of lettuce roots. Egg-mass colonization with *V. chlamydosporium* varied from 16% to 43% when soil was infested with 8 *M. hapla* eggs and treated with 5,000 or 10,000 chlamydospores of *V. chlamydosporium*/cm^3 soil. This treatment resulted in fewer J2 entering roots of bioassay lettuce seedlings planted in the infested soils after harvesting the first lettuce plants 7 weeks after infestation with *M. hapla. Hirsutella rhossiliensis* (0 to 4.3 colonies/cm^3 soil), *V. chlamydosporium* (500 to 10,000 chlamydospores/cm^3 soil), or their combination, added to organic soils with 8 *M. hapla* eggs/cm^3 soil, generally did not affect lettuce weight, root galling, or egg production of *M. hapla.* However, when lettuce was replanted in a mix of infested and uninfested soil (1:3 and 1:7, v:v), egg production was lower in soils with *V. chlamydosporium* than in soils without the fungus. Both fungi have potential to reduce the *M. hapla* population, but at densities below 8 eggs/cm^3 soil.

Key words: biological control, *Hirsutella rhossiliensis, Lactuca sativa,* lettuce, *Meloidogyne hapla,* nematode, northern root-knot nematode, organic soil, *Verticillium chlamydosporium.*

The northern root-knot nematode (*Meloidogyne hapla* Chitwood) is one of the major constraints of lettuce (*Lactuca sativa* L.) production on organic (muck) soils in New York. Severely galled roots of infected plants are unable to provide sufficient water and nutrients for normal lettuce growth. Lettuce plants heavily infected with *M. hapla* produce small and loose heads that are unmarketable. Soil fumigation with nematicides, the standard treatment for control of the northern root-knot nematode in commercial lettuce production, is costly, difficult to apply, gives inconsistent results, and is hazardous for the environment and human health. Public concern for the quality of the environment and food safety, as well as legislation aimed at reducing the use of soil fumigants, have increased the need for alternative control measures for plant-parasitic nematodes (Noling and Becker, 1994). One management tool that could reduce nematode damage to crops is the application of biological control agents.

Hirsutella rhossiliensis Minter & Brady and *Verticillium chlamydosporium* Goddard are among the most studied biological control agents of plant-parasitic nematodes. *Hirsutella rhossiliensis* attacks vermiform nematodes, and *V. chlamydosporium* parasitizes eggs of root-knot and cyst nematodes (Kerry and Jaffee, 1997). Spores of *H. rhossiliensis* produced on flask-shaped phialides are coated with an adhesin and stick readily to the cuticles of passing nematodes. Germinating spores penetrate the cuticle, and hyphae are formed in the body cavity. When the cadaver is colonized, hyphae grow out and sporulate when they are aerial (not submerged in water) (Jaffee and Zehr, 1982; Jaffee et al., 1990). The fungus has been studied as a parasite of *Mesocriconema xenoplax* (Eayre et al., 1987; Jaffee and Zehr, 1982, 1983), *Pratylenchus penetrans* (Timper and Brodie, 1993, 1994), *Heterodera schachtii,* and *M. javanica* (Jaffee and Muldoon, 1989; Lackey et al., 1994; Tedford et al., 1992, 1993, 1995). *Hirsutella rhossiliensis* was a poor

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[1] Former Graduate Research Assistant and Professor, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456. Present address of first author: Agricultural Experiment Station, Department of Crop Protection, Burg. Van Gansberghelaan 96, B-9820 Merelbeke, Belgium.
E-mail: n.viaene@clo.fgov.be
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competitive saprophyte and depended on the presence of nematodes for survival (Jaffee and Zehr, 1985; Jaffee et al., 1992).

Verticillium chlamydosporium infects nematode eggs and sedentary females of cyst nematodes by hyphae produced on actively growing mycelium (Kerry and de Leij, 1992). Survival and spread of the fungus occur through chlamydospores, microconidia, and mycelium (Kerry and Jaffee, 1997). *Verticillium chlamydosporium* colonizes the rhizosphere, which facilitates the infection of egg masses protruding from female root-knot nematodes on infected roots (de Leij and Kerry, 1991). The degree of rhizosphere colonization varies with host plant and isolate of *V. chlamydosporium* (Bourne et al., 1996; Kerry and de Leij, 1992; Kerry et al., 1993). *Verticillium chlamydosporium* has been studied extensively as a potential biological control agent of *Heterodera* spp. and *Meloidogyne* spp. (Kerry and Jaffee, 1997). One isolate of the fungus suppressed the population of *M. hapla* on tomato plants by more than 90% in microplots filled with sandy loam (de Leij et al., 1993). In contrast with *H. rhossiliensis, V. chlamydosporium* is a facultative parasite and a good saprophytic survivor in the soil. The latter fungus established better in peaty sand than in loamy sand or sand (de Leij et al., 1993). The objective of this research was to investigate the potential of *H. rhossiliensis* and *V. chlamydosporium* as biological control agents for *M. hapla* on lettuce grown in organic soil. A summary of the results was previously reported (Viaene and Abawi, 1995).

Materials and Methods

Plants, soil, and greenhouse conditions: Seeds of lettuce cv. Montello were pre-germinated in petri dishes on moist filter paper for 3 to 7 days before planting. The organic soil used in all experiments was obtained from a commercial lettuce field near Oswego, New York. It was a Carlisle Muck soil (up to 75% organic matter) with a pH of 4.7 and a bulk density of 0.59 g/cm^3. The infestation of *M. hapla* in this soil was below the detection efficiency of soil bioassays. Neither of the fungi studied was detected in the soil. Soil was used without any treatment, except for the experiment on spore transmission of *H. rhossiliensis,* for which soil was autoclaved. Experiments were conducted in the greenhouse where the temperature varied between 24 and 27 °C. Lettuce plants were watered daily and fertilized weekly with a complete fertilizer solution (NPK 16-32-16), and insect infestations were controlled as needed.

Nematode inoculum and evaluation of nematode damage to lettuce. Populations of *M. hapla* were maintained on tomato (*Lycopersicon esculentum* cv. Rutgers) in the greenhouse. Nematode eggs were extracted from tomato roots with a sodium hypochlorite solution (Hussey and Barker, 1973). Soil infestation with *M. hapla* in all the experiments was performed by pipeting nematode eggs in an aqueous suspension on the soil surface at a rate of 8 eggs/cm^3 soil, unless mentioned otherwise. This infestation level is the estimated damage threshold of *M. hapla* to lettuce grown in organic soil in the greenhouse (Viaene and Abawi, 1996). Nematode damage to lettuce was evaluated at harvest time by weighing the lettuce plants, assessing the severity of root galling caused by *M. hapla,* and extracting and counting nematode eggs from the roots. Root-galling severity was rated on a scale from 1 (no galling) to 9 (>80% of the root system galled). Ratings of 2 to 8 indicated that 1–3%, 4–10%, 11–25%, 26–35%, 36–55%, 56–65%, and 66–80% of the root system were galled, respectively. Nematode eggs were extracted from lettuce roots with a sodium hypochlorite method (Hussey and Barker, 1973), except that roots were shaken for 15 rather than 4 minutes.

Fungal inoculum and evaluation of fungal soil colonization and nematode parasitism: An isolate of *Hirsutella rhossiliensis* (IMI 265748) was provided by B. A. Jaffee. The fungus was grown on 50% potato-dextrose agar (1/2 PDA) at 22 °C for 3 weeks to allow for adequate sporulation. Since detached spores are unable to infect nematodes (McInnis and Jaffee, 1989), test soil was treated with either *H. rhossiliensis*-infected second-stage

juveniles (J2) of *M. hapla* or vegetative colonies of *H. rhossiliensis*. Both inoculum sources, which consist of assimilative hyphae, are known to produce phialides and infective spores in soil (Jaffee et al., 1990; Lackey et al., 1992). *Hirsutella rhossiliensis*-infected J2 were obtained by adding healthy J2 of *M. hapla* to sporulating cultures of *H. rhossiliensis* for 2 days. The healthy J2 were obtained from Baermann funnels where eggs of *M. hapla* had been pipeted onto autoclaved sand to hatch, after they had been disinfested in 0.001% merthiolate for 30 minutes and rinsed in sterile distilled water. A suspension of infected J2 was obtained by rinsing the culture plates with sterile distilled water. More than 90% of the collected J2 were infected with the fungus. Vegetative colonies (pellets) of *H. rhossiliensis* were prepared in shake culture (Lackey et al., 1992). They were added to soil within 24 hours after retrieval from the potato-dextrose broth without drying. Their fresh weight varied per batch and is given for each test. The presence of *H. rhossiliensis* in soils was checked by filling 25-cm^3 vials with samples of the soils and incubating them at 22 °C for 7 to 10 days in a plastic box with moist paper towels at the bottom, to allow for growth and spore production of *H. rhossiliensis*. About 500 assay J2, acquired by hatching disinfested eggs on Baermann funnels, were then added to each vial. Assay nematodes were extracted from the vials with centrifugal flotation 4 days later (Jenkins, 1964). For each vial, between 10 and 25 retrieved nematodes were observed with a microscope at ×400, and the percentage of J2 that exhibited sporulation or had spores attached to their cuticles was recorded.

Isolate 10 of *V. chlamydosporium* (CMI cc 334168) was provided by B. R. Kerry. Cultures of the fungus were grown on 1.5% water agar at 25 °C for 2 weeks before they were used in the in-vitro test or as an inoculum for sand-barley cultures (de Leij et al., 1993). Soils were treated by pipeting a suspension of chlamydospores, harvested from the sand-barley cultures, on the soil surface of the pots. The colonization of nonrhizosphere soil with *V. chlamydosporium* was measured by taking a soil sample between the plant stem and the edge of the pot, and spreading serial dilutions of the soil on semi-selective medium (de Leij and Kerry, 1991). One soil sample was taken per pot with a 7-mm-diam. cork borer, and serial dilution series were made with 1-g subsamples using the method described by Kerry et al. (1993). Aliquots of 0.5 ml were spread on the semi-selective medium in 9-cm petri dishes, repeating each dilution three times. Plates were incubated for 10 days at 25 °C before colony-forming units (CFU) of *V. chlamydosporium* were counted. These samples of nonrhizosphere soil were taken at regular intervals to monitor the establishment of *V. chlamydosporium*. Estimation of fungal densities in rhizosphere soil (soil adhering to the roots) was investigated at the harvest of four experiments. Rhizosphere soil was collected by shaking at least four freshly harvested root systems per treatment for 2 minutes in a paper bag, to remove the soil still adhered to the roots. This soil was sieved through a 250-µm-pore sieve to discard root pieces, and a 1-g subsample per root system was used to prepare a soil-dilution series, which was then spread on semi-selective medium as described above.

In all experiments with *V. chlamydosporium*, the percentage of *M. hapla* egg masses that became colonized with the fungus was determined. Egg masses (usually 10) were picked from the root system of each harvested lettuce plant, rinsed in sterile distilled water, and incubated on water agar amended with streptomycin sulfate (200 mg/liter) at 25 °C for 10 to 14 days. Egg masses showing sporulating mycelium of *V. chlamydosporium* were considered to contain infected eggs. The influence of this colonization on the population density of *M. hapla* was evaluated with a bioassay conducted after the lettuce was harvested. The bioassay consisted of planting 5 to 7-day-old lettuce seedlings in 120-ml plastic cups (1 seedling/cup) filled with samples of the soils treated with the various densities of *V. chlamydosporium* included in the test. Cups were infested with *M. hapla* at planting of the first lettuce crop. After 8 to 14 days in a growth chamber

at 24 °C, the seedling roots were washed and stained and J2 in the roots were counted (Byrd et al., 1983).

Sporulation of H. rhossiliensis from M. hapla: Disinfested eggs of *M. hapla* were pipeted onto 3-week-old sporulating cultures of *H. rhossiliensis* on ½ PDA. After 2 days, hatched J2 were picked up at random with a needle and transferred to incubation dishes. An incubation dish consisted of a petri dish with streptomycin-sulfate amended water agar (200 mg/liter), from which 10 agar discs (1-cm diam.) were removed with a cork borer (Jaffee et al., 1990). One nematode in a drop of sterile distilled water was placed in each well, and the incubation dishes were kept at 22 °C. Juveniles also were transferred from the cultures of *H. rhossiliensis* to four petri dishes with streptomycin-sulfate amended water agar (20 nematodes/plate) without wells, solely to determine the percentage of J2 that became infected with *H. rhossiliensis*. The test was repeated with a different batch of eggs of *M. hapla*. The number of spores of *H. rhossiliensis* produced per infected J2 was counted after 1, 3, 5, 8, 11, and 14 days in test 1. In test 2 (the repeat test), spores also were counted on days 17 and 20. Spores were directly observed on the hyphae with a dissecting microscope at ×40. A total of 24 and 8 J2 were observed in tests 1 and 2, respectively, for each incubation period.

Spore transmission of H. rhossiliensis in organic soil: To test the ability of *H. rhossiliensis* spores to adhere to the cuticles of *M. hapla* J2 and infect the nematodes in the soil environment, a spore-transmission test was performed in organic soil. The soil had been stored at 10 °C for at least 9 months before it was used in the tests. Capped, 25-cm^3 plastic vials, each with six drainage holes in the bottom, were filled with 20 cm^3 of untreated or autoclaved (1 hour at 121 °C) organic soil. Inoculum of *H. rhossiliensis* was added to the vials as an aqueous suspension of infected *M. hapla* J2 (250 J2/vial, test 1) or as vegetative colonies of the fungus placed in the middle of each vial (12 colonies/vial, test 2). Both sources of *H. rhossiliensis* were obtained as described above. Vials were incubated in a plastic box containing moistened paper towels at 22 °C for 10 days (test 1) or 1 month (test 2), to allow for the sporulation of *H. rhossiliensis*. After this incubation, 400 assay nematodes (1 to 5 days old) were added to each vial in 2.75 ml (test 1) or 1.5 ml (test 2) sterile distilled water. The assay nematodes were extracted after 4 days (test 1) or 5 days (test 2) and examined for spore attachment at ×400. Between 9 and 47 J2/vial were examined in test 1, and 15 J2/vial were examined in test 2. There were four replicate vials for each treatment (untreated or autoclaved soil), and two control treatments (J2 added to either autoclaved or untreated soil without *H. rhossiliensis*) were included in each test.

Effect of H. rhossiliensis on penetration of M. hapla juveniles in lettuce roots: Five similar tests were performed to evaluate the effect of several soil application rates of *H. rhossiliensis* (0 to 3.5 vegetative colonies/cm^3 soil in 200-cm^3 pots) on the penetration of *M. hapla* J2 into roots of lettuce seedlings. Each application rate was repeated 8 to 10 times. Vegetative colonies (0.4 to 1.5 mg/colony) for each test were obtained from different batches of fungal shake cultures. Colonies were resuspended in about 10 ml water, spread over the soil surface in each pot, and covered with 1 cm of soil. Pots were kept in the greenhouse and watered regularly during 1 week, to allow for growth and sporulation of the fungus. Soils were infested with *M. hapla,* and a lettuce seedling was planted in each pot 3 days later. After 10 days, the roots of the lettuce seedlings were stained and the number of *M. hapla* J2 inside the roots were counted (Byrd et al., 1983). After the seedlings were removed, a portion of the soil of the various treatments was assayed for the presence of *H. rhossiliensis,* as described above.

Effect of H. rhossiliensis on reproduction of M. hapla and its damage to lettuce: Vegetative colonies of *H. rhossiliensis* (0.50 ± 0.06 mg/colony) were added to clay pots (500 cm^3) filled with organic soil at a rate of 0, 0.06, 0.3, 0.6, 1.4, or 2.8 colonies/cm^3 soil. Pots were infested with *M. hapla* the following day. A control treatment consisting of un-

treated soil (without addition of nematodes and fungi) was included. Each treatment was replicated eight times, and one lettuce seedling was planted per pot 1 week after the nematode infestation. In a similar test, organic soil was placed in 300-cm^3 pots and treated with 0, 2.3, or 4.3 colonies/cm^3 soil (1.28 ± 0.08 mg/colony). After 1 week in the greenhouse, the soil in each pot was infested with *M. hapla* and planted to lettuce. Untreated soil served as a control, and all treatments were replicated 11 times. The same treatments of this repeat test also were established in 120-cm^3 plastic cups, with nine replicate cups per treatment. Lettuce seedlings were removed from the cups after 10 days, and the number of *M. hapla* J2 that had penetrated the roots was counted. Lettuce plants of both pot tests were harvested 8 weeks after planting and evaluated as described above.

In-vitro parasitism of M. hapla by V. chlamydosporium: Egg masses were disinfested in 0.525% NaOCl solution for 30 seconds, rinsed three times in sterile distilled water, and then added to sporulating cultures of *V. chlamydosporium* on 1.5% water agar. After 24 hours, 50 egg masses were transferred to petri dishes with 1.5% water agar (10 egg masses/petri dish) and incubated at 25 °C for 2 weeks. A petri dish with egg masses unexposed to *V. chlamydosporium* served as a control. The number of egg masses exhibiting mycelium of *V. chlamydosporium* was counted, and the eggs of one egg mass from each plate were inspected for infection by *V. chlamydosporium* at ×400.

Parasitism of M. hapla by V. chlamydosporium in soil and its effect on lettuce: Organic soil in clay pots (300 cm^3) was treated with *V. chlamydosporium* at a rate of 0, 500, 5,000, or 10,000 chlamydospores/cm^3 soil. A lettuce seedling was planted in each pot the following day. Twenty days later, pots were infested with *M. hapla* at a rate of 2, 4, or 8 eggs/cm^3 soil. Each combination of the fungal and nematode inocula was repeated five times, and lettuce plants were harvested and evaluated 8 weeks after planting. In addition, root systems of the same treatment were mixed and 18 to 24 egg masses/treatment, incubated in two petri dishes, were inspected for colonization with *V. chlamydosporium* as described above.

Efficacy of V. chlamydosporium as a biological control agent of M. hapla in soil: Pots (300 cm^3) were filled with organic soil and treated with 0, 5,000, or 10,000 chlamydospores of *V. chlamydosporium*/cm^3 soil. Lettuce was planted in all pots 1 day after fungal treatment, and *M. hapla* eggs were added 2 weeks later. The test was repeated, and each treatment was replicated 14 times in the first test and 16 times in the repeat test. A control treatment of untreated soil (neither nematodes nor fungi added) was included in each test. The effects of *V. chlamydosporium* on the population of *M. hapla* and its damage to lettuce were investigated in two consecutive lettuce plantings. At the first harvest, 7 weeks after infestation with *M. hapla,* lettuce was weighed and roots of half of the pots were used to evaluate severity of root galling, to extract and count the nematode eggs, and to determine the percentage of egg masses colonized with *V. chlamydosporium.* In addition, a bioassay with lettuce seedlings was performed in the soils of the various treatments of these pots. Soil and roots of the other half of the pots were thoroughly mixed, returned to the pots, and replanted to lettuce 10 days later in the first test. Soil and roots of half of the pots of the repeat test were first mixed and then diluted with uninfested organic soil at a ratio (v:v) of 3:1 or 7:1 before returning to the pots. The resulting 25%- and 12.5%-infested soils were planted to lettuce 2 weeks later. The second lettuce planting was harvested 8 weeks after planting, and the same data were taken as at the first harvest. The abundance of *V. chlamydosporium* in the soil was measured every 2 to 3 weeks by placing serial dilutions of the soil on semi-selective medium.

Control of M. hapla on lettuce with a combination of H. rhossiliensis and V. chlamydosporium: The growth interaction between *H. rhossiliensis* and *V. chlamydosporium* was investigated in vitro at 22 °C. Plugs of *H. rhossiliensis* and *V. chlamydosporium* were placed alone or together (4 cm apart) in 9-cm-diam. petri dishes containing 1.5% water agar (WA),

half-strength potato dextrose agar (1/2 PDA), or 1% malt agar (MA). The colony diameters of the two fungi were measured at regular intervals over 40 days in three replicate plates per combination.

A greenhouse test was performed in 300-cm^3 pots filled with organic soil treated with 5,000 chlamydospores of *V. chlamydosporium*/cm^3 soil and 2.3 colonies of *H. rhossiliensis*/cm^3 soil (0.4 mg per colony), singly or in combination. Pots were planted with two lettuce seedlings, and soil was infested with *M. hapla* 10 days later. Two fungus-free control treatments consisting of lettuce grown in uninfested soil and in nematode-infested soil were included. At the beginning of the experiment, a bioassay with lettuce seedlings was performed in eight replicate cups filled with the *H. rhossiliensis*-treated soils and *M. hapla*-infested control, to assess the efficacy of the fungus. Seedlings of this bioassay were stained 10 days after nematode infestation. In the greenhouse pot test, there were 20 replicate pots/soil treatment, and lettuce was harvested 8 weeks after soil infestation with *M. hapla*. Half of the pots were used to assess nematode damage to lettuce and control of *M. hapla* by the two fungi, measuring the same parameters as in the above-described greenhouse test. A second lettuce crop was planted in the soil of the other half of the pots, either in 100% (undiluted), 25%, or 12.5%-infested soil, replicated 5, 8, and 8 times, respectively. The 25% and 12.5%-infested soil preparations were obtained by mixing the soil from the first lettuce crop with uninfested organic soil at proportions of 3:1 and 7:1, respectively. The second lettuce crop was harvested 8 weeks after planting and data were taken as described above, except that no bioassay was performed in the 25%-infested soil.

Establishment of *V. chlamydosporium* in the soils treated with the fungus, alone and in combination with *H. rhossiliensis*, was measured every 2 to 3 weeks by plating out serial dilutions on semi-selective medium. This was also done for the control treatment infested with *M. hapla* and for the soil of an extra series of eight pots treated with *V. chlamydosporium* but left unplanted. At each harvest, soils treated with *H. rhossiliensis* at the beginning of the experiment were put in vials (5 per treatment) and assayed for the fungus.

Statistical analysis: Regression was used to describe the relation between the number of vegetative colonies of *H. rhossiliensis* and the suppression of root penetration by *M. hapla* J2. The number of colonies necessary for a 50% reduction in root penetration was estimated with statistical calibration (inverse regression or inverse prediction) (Neter et al., 1990). Pot tests were arranged as randomized complete block designs, and data for lettuce weight, root galling severity, and numbers of *M. hapla* eggs were analyzed with ANOVA, using the LSD test for mean separation ($P = 0.05$). Numbers of *M. hapla* eggs were log-transformed before analysis. Nonparametric methods (Mann-Whitney test) were used when the data did not meet the ANOVA requirements (e.g., for percentages of egg masses colonized by *V. chlamydosporium* and for numbers of J2 in bioassay seedlings). Growth rates of the two fungi grown singly or in combination were compared by testing the homogeneity of the regression coefficients of the linear regression functions relating colony diameter and incubation time. This was done separately for the three different media.

Results

Sporulation of H. rhossiliensis from M. hapla: Spore production on hyphae of *H. rhossiliensis* emerging from colonized nematodes started 3 days after transfer of the infected *M. hapla* J2 to the incubation dishes. An average of 124 ± 9 spores were produced per J2 over 14 days in test 1 ($n = 24$) (Fig. 1). In test 2 ($n = 8$), 78 ± 21 new spores were produced per J2 over a period of 17 days. The infection of *M. hapla* J2 was quite successful in the first test, as hyphae of *H. rhossiliensis* emerged from the bodies of 85% of the nematodes. Only 56% of the nematodes were infected with *H. rhossiliensis* in the second test.

Spore transmission of H. rhossiliensis in organic soil: It was demonstrated that *H.*

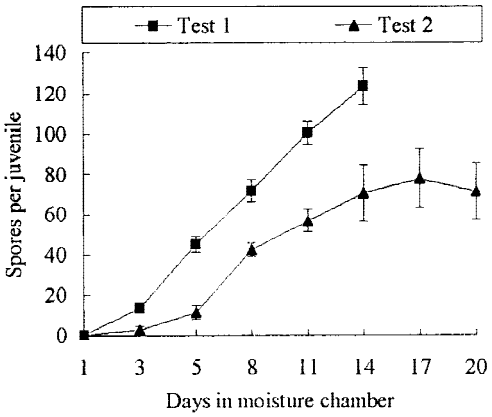

FIG. 1. Spore production of *Hirsutella rhossiliensis* from infected *Meloidogyne hapla* juveniles (J2) in incubation dishes at 22 °C. Each point is the mean ± SE of 24 (test 1) or 8 (test 2) J2.

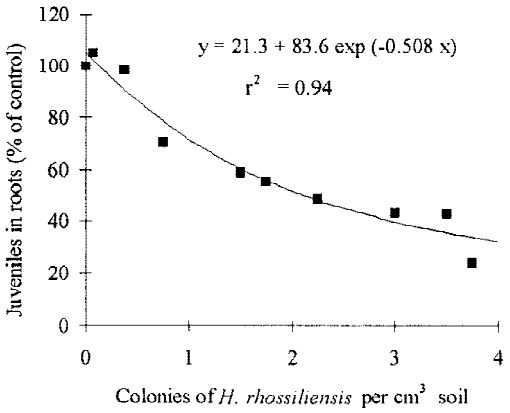

FIG. 2. Effect of the application rate of *Hirsutella rhossiliensis* on penetration of lettuce roots by *Meloidogyne hapla* J2 in soil infested with 8 eggs/cm^3 soil. Each point represents the mean of 8 to 46 seedlings examined. Data of five experiments were combined for exponential regression.

rhossiliensis infected and reproduced on *M. hapla* J2 in organic soil. The percentage of recovered assay *M. hapla* J2 with adhering spores of *H. rhossiliensis* was the same whether the soil had been autoclaved or left untreated. In the test where *H. rhossiliensis*-infected J2 were applied to the soil (12.5 J2/cm^3 soil), 16 ± 4% and 15 ± 6% of the recovered assay nematodes had spores of *H. rhossiliensis* attached to them in the autoclaved and untreated soil, respectively. When colonies of *H. rhossiliensis* were applied (0.6 colonies/cm^3 soil), 38 ± 5% and 35 ± 7% of the recovered assay nematodes, from autoclaved and untreated soil, respectively, were observed with adhering spores. None of the nematodes retrieved from the controls (soils without application of *H. rhossiliensis*) had spores of the fungus attached to them.

Effect of H. rhossiliensis on penetration of M. hapla J2 in lettuce roots: The numbers of J2 that entered the roots of lettuce seedlings decreased exponentially ($P < 0.001$) with increasing numbers of vegetative colonies of *H. rhossiliensis* added to the soil (Fig. 2). A regression was performed with the means of all five tests (Fig. 2), as there was no evidence that the regression differed between the tests. Between 35 and 143 J2 entered the roots in the control treatment (soil without *H. rhossiliensis*). Using statistical calibration, it was calculated that 1.9 colonies of *H. rhossiliensis*/cm^3 soil were required to obtain a 50% decrease in root penetration by J2 in a soil infested with 8 *M. hapla* eggs/cm^3 soil. Presence of *H. rhossiliensis* in soil was confirmed as assay nematodes added to the soils were found to be infected with the fungus. The percentage of J2 with at least one adherent spore of *H. rhossiliensis* varied between 4% and 39%. Spore transmission was greater in the soils treated with the higher numbers of vegetative colonies of *H. rhossiliensis* (data not shown).

Effect of H. rhossiliensis on reproduction of M. hapla and its damage to lettuce, alone or in combination with V. chlamydosporium: The effect of different application rates of *H. rhossiliensis* on root galling and nematode reproduction was variable, and none of the soil applications of *H. rhossiliensis* affected lettuce weight (Table 1). However, root-galling severity was reduced by the highest application levels of *H. rhossilienis* in the first test (Table 1). Significantly fewer J2 penetrated the roots of lettuce seedlings planted in the soil that received the highest application rate in test 2 (4.3 colonies/cm^3 soil) compared with the treatment without *H. rhossiliensis,* but this did not result in significantly less root galling or in lower egg production at harvest time (Table 1). Similarly, there was a 50% reduction in J2 penetration

TABLE 1. Effect of soil density of *Hirsutella rhossiliensis* (Hr) on reproduction of *Meloidogyne hapla* (Mh) and its damage to lettuce in organic soil.

Soil treatment		*M. hapla*[a]			
Hr colonies per cm^3 soil	Mh[b]	Juveniles per seedling[c]	Eggs per root	Root-gall rating[d]	Weight of lettuce (g)
Test 1:					
0	+		37,900	8.0	13.1
0.06	+		34,400 ns	6.5*	14.8 ns
0.3	+		33,400 ns	7.8 ns	14.7 ns
0.6	+		45,800 ns	7.6 ns	15.5 ns
1.4	+		15,300*	5.4**	15.3 ns
2.8	+		35,900 ns	6.3**	15.3 ns
0	–		—	—	14.5 ns
LSD			20,500	1.25	3.3
n			4	8	8
Test 2:					
0	+	89.7	71,500	6.6	29.1
2.3	+	76.0 ns	69,500 ns	7.1 ns	28.4 ns
4.3	+	43.1*	63,800 ns	5.6 ns	31.7 ns
0	–	—	—	—	37.0*
LSD		36.7	31,300	2.1	6.6
n		9	11	11	11

[a] Lettuce was planted immediately after addition of *H. rhossiliensis* (Hr) to the soil and was harvested 8 weeks later. Means in each column were compared with the mean of the control treatment where no *H. rhossiliensis* was applied in *M. hapla* (Mh)-infested soil, using the LSD test.

[b] Soil was infested with 8 *M. hapla* eggs/cm^3 soil, 1 day (test 1) or 1 week (test 2) after addition of *H. rhossiliensis*.

[c] Number of juveniles inside roots of lettuce seedlings 10 days after planting in cups filled with the same soil treatments as those in the pots.

[d] Root-galling severity was evaluated on a scale from 1 to 9 where 1 = 0%, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–35%, 6 = 36–55%, 7 = 56–65%, 8 = 66–80%, 9 = >80% of the root system galled.

in lettuce seedlings in soils treated with *H. rhossiliensis* (2.3 colonies/cm^3 soil) in the experiment where *H. rhossiliensis* was combined with *V. chlamydosporium,* at the beginning of the experiment (data not shown). Results of bioassays, performed when the first and second lettuce plantings in undiluted soils were harvested, did not show a difference in J2 penetration of roots of lettuce seedlings planted in untreated soil and soils treated with one or both of the nematophagous fungi. At the harvest of lettuce planted in 12.5%-diluted soil, however, significantly fewer J2 were found in lettuce seedlings planted in soils with *H. rhossiliensis* (23.9 J2/seedling), *V. chlamydosporium* (19.0 J2/seedling), or both fungi (34.1 J2/seedling) than in seedlings planted in soil of the control treatment (60.7 J2/seedling). In the same test with both fungi, application of *H. rhossiliensis,* alone or in combination with *V. chlamydosporium,* had no effect on lettuce weight, egg production of *M. hapla* on lettuce roots, or degree of root galling. After the first lettuce harvest, 27% of assay nematodes were infected with *H. rhossiliensis* in the soil initially treated with only *H. rhossiliensis.* In contrast, *H. rhossiliensis* could not be detected in the soil treated with *H. rhossiliensis* and *V. chlamydosporium* together, nor in any of the soil treatments after the second lettuce harvest. In-vitro growth of *V. chlamydosporium* and *H. rhossiliensis* was not affected when they were grown together on WA. However, on ½ PDA and on 1% MA, *H. rhossiliensis* grew slower in the presence of *V. chlamydosporium* than in pure culture ($P < 0.05$) (Fig. 3).

Effect of V. chlamydosporium on reproduction of M. hapla and its damage to lettuce, alone or in combination with H. rhossiliensis: Strain 10 of *V. chlamydosporium* colonized 78% of the egg masses of the New York population of *M. hapla.* Mycelium of *V. chlamydosporium* was observed inside 87% of the eggs obtained from colonized egg masses. *Verticillium chlamydosporium* became established and survived in organic soil throughout the course

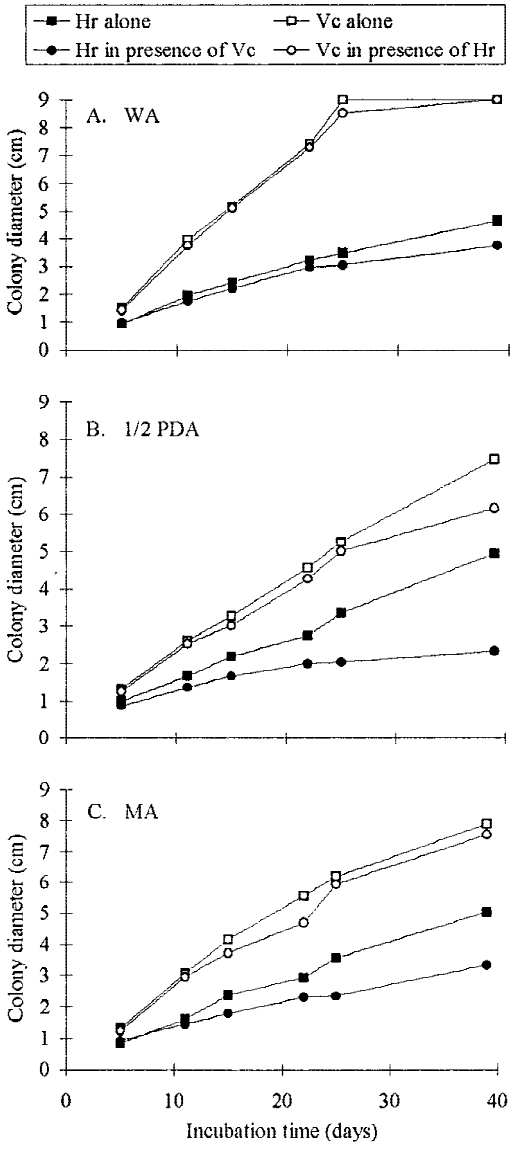

FIG. 3. Growth of *Verticillium chlamydosporium* (Vc) and *Hirsutella rhossiliensis* (Hr) on various media in 9-cm-diam. petri dishes incubated at 22 °C. A) 1.5% water agar. B) Half-strength potato-dextrose agar. C) 1% malt agar.

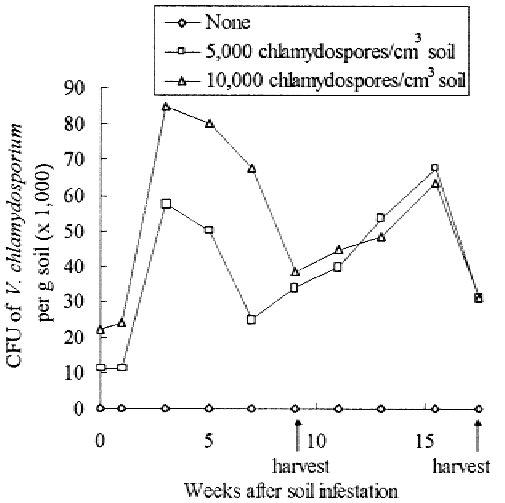

FIG. 4. Colony-forming units (CFU) of *Verticillium chlamydosporium* in soils treated with 0, 5,000, or 10,000 chlamydospores/cm^3 soil, without or with *Meloidogyne hapla* (8 eggs/cm^3 soil). The soil was planted to two sequential lettuce crops in the greenhouse. First and second harvest of lettuce was at 9 and 17.5 weeks, respectively, after initial application of *V. chlamydosporium* to soil. The second lettuce was planted 10 days after harvesting the first planting.

of the three monitored experiments (Fig. 4). Data from only one experiment are shown because the change in the numbers of CFU of *V. chlamydosporium* over time followed a similar pattern in all experiments. The high application rate (10,000 chlamydospores/cm^3 soil) resulted in higher numbers of CFU per gram of soil than the low application rate (5,000 chlamydospores/cm^3 soil) during the first lettuce planting only (Fig. 4). When a second lettuce crop was planted, the soil population of *V. chlamydosporium* recovered (CFU per gram of soil) was approximately the same, regardless of the initial application level (Fig. 4). Addition of *H. rhossiliensis* to the soil resulted in an increase in the numbers of CFU per gram of soil of *V. chlamydosporium*, but only during the first lettuce planting (data not shown). *Verticillium chlamydosporium* survived at the same densities, and for at least 12 weeks, in soils left unplanted or planted to lettuce. The numbers of CFU per gram of rhizosphere soil were either approximately the same as or lower than the numbers of CFU per gram of nonrhizosphere soil (Table 2).

At application levels of 2 or 4 *M. hapla* eggs/cm^3 soil, the percentage of *M. hapla* egg masses colonized by *V. chlamydosporium* increased with increasing application levels of the fungus (Fig. 5; Table 3). However, in soils infested with 8 *M. hapla* eggs/cm^3 soil, no difference in egg-mass colonization could be detected whether 5,000 or 10,000

TABLE 2. Colonization of rhizosphere and nonrhizosphere soils with *Verticillium chlamydosporium* in pots planted to lettuce.

Sampling date[a]	Soil[b]	CFU of *V. chlamydosporium*/g soil		Replications[d]
		Pi = 5,000[c]	Pi = 10,000[c]	
Test 1, first harvest	RS	43,375	62,125	8
	NRS	353,333	366,667	1
Test 1, second harvest in 25%-diluted soil	RS	64,000	122,000	4
	NRS	132,500	165,427	1
Test 2, first harvest in soil infested with Vc only	RS	78,750		5
	NRS	40,000		1
Test 2, first harvest in soil infested with Vc and Hr	RS	51,000		5
	NRS	60,000		1

[a] In test 1, soil was treated with *V. chlamydosporium* at two densities (Pi) and lettuce was planted for two crop cycles. The soil was diluted to 25% with untreated soil before the second lettuce was planted. In test 2, soil was treated with *V. chlamydosporium* and *H. rhossiliensis*, individually and combined.

[b] RS and NRS refer to rhizosphere and nonrhizosphere soil, respectively.

[c] Pi = initial application rate of *V. chlamydosporium* (number of chlamydospores per cm^3 soil).

[d] Each replication of a rhizosphere soil sample was taken from a different root system. Samples of nonrhizosphere soil were taken from 8 to 10 replicate pots of the same soil treatment and mixed. A 1-g subsample of this mixture was used in preparing the soil dilutions for plating on semi-selective medium.

chlamydospores had been added to the soil (Fig. 5; Tables 4, 5). Between 15.5% (Table 4) and 42.7% (Table 5) of the egg masses on roots of a first lettuce planting in soil infested with 8 of *M. hapla* eggs/cm^3 soil became colonized with *V. chlamydosporium*. Replanting lettuce in the same soil (undiluted) did not result in higher colonization levels of the egg masses on the roots of the second lettuce crop (Table 4). This was also observed in the test where *V. chlamydosporium* was applied singly and together with *H. rhossiliensis*. However, in one of the two experiments where lettuce was replanted in soil diluted with uninfested soil, up to 69.2% of the egg masses of *M. hapla* produced on lettuce roots were found to be colonized by *V. chlamydosporium* (Table 5). Addition of *H. rhossiliensis* did not affect the incidence of egg masses colonized by *V. chlamydosporium*.

The numbers of *M. hapla* J2 inside roots of lettuce seedlings planted after harvesting lettuce were 43% to 71% lower in soils treated with *V. chlamydosporium* than in soils without fungal application (Tables 4,5). There was no difference between the numbers of J2 inside roots of seedlings grown in soils treated with 5,000 or 10,000 chlamydospores of *V. chlamydosporium*/cm^3 soil (Tables 4,5). In general, *V. chlamydosporium* had little effect on the severity of root galling (Tables 3–5; Fig. 5). In the experiments where lettuce was grown for a second cycle in a diluted soil, egg production of *M. hapla* on roots of the second lettuce crop tended to be less in soils with *V. chlamydosporium* than in soils without the fungus. This reduction, however, was significant only in the 12.5%-diluted soil treated with 5,000 chlamydospores/cm^3 soil (Table 5). In the test where both fungi were combined, between 0 and 53% fewer eggs were produced when *V. chlamydosporium* was added to the soil compared with egg production in soil without fungal treatment. However, none of the reductions was significant. There was no effect of soil treatment with *V. chlamydosporium* on the weight of the first lettuce crop (Fig. 5A; Tables 3–5). The weight of the second lettuce crop planted in undiluted soil treated with *V. chlamydosporium,* however, was higher than that of lettuce planted in soil infested only with *M. hapla* (Table 4).

DISCUSSION

Efficacy of spore transmission in soil and sporulation rate of *H. rhossiliensis* are important attributes determining the potential of the fungus to act as a biological control agent against the target host nematode. Spore production of *H. rhossiliensis* per *M. hapla* J2 (around 100) was comparable to spore production reported previously on *H.*

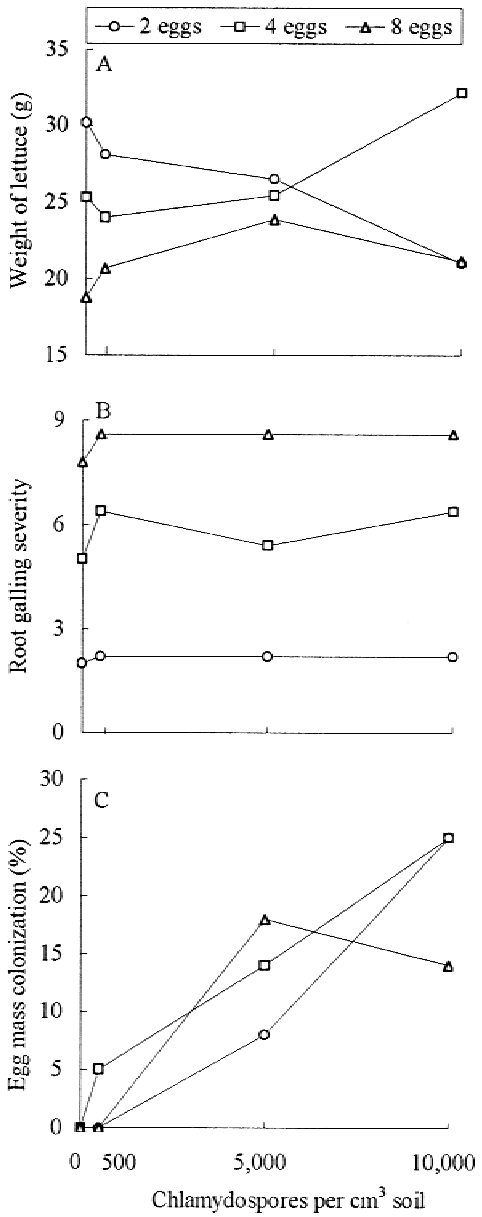

FIG. 5. Effects of different combinations of initial soil application densities of *Meloidogyne hapla* (2, 4, and 8 eggs/cm^3 soil) and *Verticillium chlamydosporium* (0, 500, 5,000, and 10,000 chlamydospores/cm^3 soil) on A) weight of lettuce, B) severity of root galling, C) colonization of egg masses of *M. hapla* by *V. chlamydosporium.* Values are means of five replicates, except for the percentages of colonized egg masses, which are based on 18 to 24 egg masses per treatment. Root-galling severity was evaluated on a scale from 1 to 9 where 1 = 0%, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–35%, 6 = 36–55%, 7 = 56–65%, 8 = 66–80%, 9 = >80% of the root system galled. Statistical analysis of the data is shown in Table 3.

schachtii J2 (112 spores) (Jaffee et al., 1990). These spore production levels are much lower than that reported on *C. xenoplax* (about 700 spores), the nematode from which the fungal isolate was obtained (Jaffee and Zehr, 1983), possibly due to the greater volume of *C. xenoplax* compared to that of *M. hapla* or *H. schachtii* J2. The origin of the isolate of *H. rhossiliensis* did not influence the virulence of the fungus to other nematode species (Tedford et al., 1994). Whether vegetative colonies or colonized host nematodes were used as a source of the fungus, spore transmission of *H. rhossiliensis* in organic soil was not hampered by microorganisms, as the percentage of *M. hapla* J2 with at least one attached spore was the same in autoclaved and untreated soils. A similar observation was made for spore transmission of *H. rhossiliensis* to *H. schachtii* in loamy sand treated with vegetative colonies of *H. rhossiliensis* (Lackey et al., 1992).

The adherence of *H. rhossiliensis* spores to *M. hapla* J2 resulted in infection and death of the nematodes, as suggested by the lower invasion of lettuce seedling roots by *M. hapla* J2 in *H. rhossiliensis*-treated soils than in untreated soils. The incubation time (7 to 10 days) provided before the planting of lettuce allowed for the sporulation of vegetative colonies of *H. rhossiliensis,* as spore formation from colonies was reported to start within 2 days and continue for at least 3 weeks in incubation dishes (Lackey et al., 1992). Additional sporulation from infected cadavers of *M. hapla,* starting within 3 days after infection and continuing for up to 17 days, probably contributed to the infection of newly hatched J2. An exponential decrease in penetration of J2 into seedling roots with increasing application rate of *H. rhossiliensis* also was observed for *H. schachtii* (Lackey et al., 1992, 1993; Tedford et al., 1994). Invasion of tomato seedlings by *M. javanica* J2 was reduced by 50% when 50 pellets of *H. rhossiliensis* were added to 100 cm^3 of loamy sand infested with egg masses of the nematode (Lackey et al., 1994). This number corresponds approximately with the 1.9 colonies/cm^3 organic soil required to suppress penetration of *M. hapla* by 50%

TABLE 3. Statistical analysis of egg-mass colonization with *Verticillium chlamydosporium,* root-gall ratings, and weight of lettuce grown under greenhouse conditions in organic soil at different densities of *Meloidogyne hapla* and *V. chlamydosporium* as shown in Figure 5.

		Mean squares		
Source of variation	df[a]	Egg masses colonized by Vc (%)[b]	Lettuce weight (g)	Root galling[c]
M. hapla (Mh)[d]	2	32.7 ns	199.5**	197.2**
V. chlamydosporium (Vc)[e]	3 or 2	547.2*	2.6 ns	2.2*
Mh × Vc	6 or 4	43.8 ns	81.7*	0.6 ns
Replications	4 or 1	72.0 ns	113.8*	1.7*
Error	44 or 9	91.9	33.7	0.7

[a] The control treatment (no. *V. chlamydosporium*) was not included in the analysis of the percentages of colonized egg masses.
[b] Between 18 and 24 egg masses of *M. hapla,* incubated in 2 replicate petri dishes/treatment, were inspected for colonization by *V. chlamydosporium.*
[c] Root galling was evaluated on a scale from 1 (no galling) to 9 (> 80% of roots galled).
[d] Nematode infestation levels were 2, 4, and 8 eggs/cm^3 soil.
[e] *V. chlamydosporium* was applied at rates of 0, 500, 5,000, and 10,000 chlamydospores/cm^3 soil.

in lettuce seedlings. A pellet contains an amount of hyphae equivalent to that contained in two vegetative colonies (Lackey et al., 1993).

Juvenile penetration into roots of assay seedlings was reduced only at the beginning of the experiments, when the nematode population density was still relatively low, or at the harvest of lettuce planted in 12.5%-diluted soil. A reduction in J2 penetration into lettuce seedlings was not observed when lettuce seedlings were planted in soils heavily infested with *M. hapla,* such as after a first lettuce crop, or after a second lettuce crop in undiluted soil. Similarly, application of *H. rhossiliensis* did not consistently result in less galling or reduced reproduction of *M. hapla.* Inability of *H. rhossiliensis* to reduce J2 invasion in seedlings and nematode populations in microplots at high nematode infes-

TABLE 4. Effect of application rate of *Verticillium chlamydosporium* (Vc) on the population of *Meloidogyne hapla* (Mh) and its damage to two sequential lettuce plantings in organic soil in the greenhouse.

Soil application rate[a]		Lettuce[b]		*M. hapla*[b]		
Vc chlamydospores per cm^3 soil	Mh	Weight (g)	Root galling[c]	Eggs per gram of root	Egg masses with Vc (%)[d]	Juveniles per seedling[e]
First planting[f]						
0	+	39.9 a	5.4 a	10,400 a	0.0 b	66.0 a
5,000	+	44.0 a	4.0 b	7,400 a	15.5 a	23.1 b
10,000	+	40.7 a	5.0 a	12,200 a	16.7 a	37.5 ab
0	–	45.2 a	1.0	—	—	—
Second planting[f]						
0	+	7.7 c	8.3 a	5,100 a	0.0 b	131.3 a
5,000	+	17.2 ab	9.0 a	7,200 a	7.4 a	38.4 b
10,000	+	14.1 b	8.9 a	3,800 a	8.8 a	47.4 b
0	–	21.8 a	1.0			

[a] *V. chlamydosporium* (Vc) was applied 1 day before lettuce planting. Two weeks later, soil was infested with 8 *M. hapla*/cm^3 soil eggs (+) or was left uninfested (–). There were seven replicate pots.
[b] Means in each column (per planting) followed by the same letter are not significantly different ($P < 0.05$) according to the least significant difference (LSD) test for lettuce weight, root-galling severity, and number of eggs per gram root, or the Mann-Whitney test for percentages of egg masses colonized by *V. chlamydosporium* and number of juveniles per seedling. Control treatments were not included in the statistical tests for the root-galling ratings.
[c] Root galling severity was evaluated on a scale from 1 (no galls) to 9 (>80% of roots galled).
[d] Average number of seven replications; each consisted of a petri dish containing 10 (first planting) or 5 to 10 (second planting) egg masses picked from the same root system.
[e] Number of juveniles in roots of 10-day-old lettuce seedlings planted in the nematode-infested soils immediately after the harvests of the first and second lettuce planting. An average of 10 assay seedlings were planted per treatment.
[f] The first lettuce planting was harvested 7 weeks after infestation with *M. hapla.* A second lettuce crop was planted 10 days later and was harvested after 8 weeks.

TABLE 5. Effect of soil application rate of *Verticillium chlamydosporium* (Vc) on *Meloidogyne hapla* (Mh) and its damage to two sequential lettuce plantings in organic soil in the greenhouse. The soil was diluted with uninfested (no nematodes, no fungi) organic soil before the second planting at the ratios (v:v) of 1:3 (25%) and 1:7 (12.5%).

Soil application rate[a]		Lettuce[b]		*M. hapla*[b]		
Vc chlamydospores per cm^3 soil	Mh	Weight (g)	Root galling[c]	Eggs per gram of root	Egg masses with Vc (%)[d]	Juveniles per seedling[e]
First planting[f]						
0	+	75.9 a	5.3 a	20,500 a	0.0 b	90.7 a
5,000	+	75.9 a	5.4 a	24,300 a	42.7 a	49.7 b
10,000	+	72.9 a	5.3 a	20,500 a	36.5 a	38.3 b
0	–	75.0 a	1.0			
Second planting (25%-infested soil)[f]						
0	+	23.3 a	7.5 a	36,900 a	0.0 b	55.3 a
5,000	+	24.8 a	7.5 a	23,600 a	69.2 a	19.7 b
10,000	+	21.0 a	7.2 a	33,300 a	62.2 a	28.8 b
0	–	23.7 a	1.0			
Second planting (12.5%-infested soil)[f]						
0	+	21.2 a	8.2 a	45,500 a	0.0 b	62.4 a
5,000	+	21.9 a	5.3 b	8,200 b	48.9 a	21.4 b
10,000	+	21.3 a	6.5 ab	21,000 a	64.3 a	23.2 b
0	–	22.7 a	1.0			

[a] *Verticillium chlamydosporium* was applied 1 day before lettuce planting. Two weeks later, soil was infested with 8 *M. hapla* eggs/cm^3 soil (+) or was left uninfested (–). There were eight replicate pots.

[b] Means in each column (per planting) followed by a common letter are not significantly different ($P < 0.05$) according to the least significant difference (LSD) test for lettuce weight, root-galling severity, and eggs per gram of root, or the Mann-Whitney test for percentages of egg masses colonized with *V. chlamydosporium* and number of juveniles per seedling. Control treatments were not included in the statistical tests for the root-galling ratings.

[c] Root-galling severity was evaluated on a scale from 1 (no galls) to 9 (>80% of roots galled).

[d] Average number of eight replications, each consisted of a petri dish containing 10 (first planting) or 5 to 10 (second planting) egg masses picked from the same root system.

[e] Number of juveniles in roots of 10-day-old lettuce seedlings planted in the nematode-infested soils immediately after the harvests of the first and second lettuce planting. An average of 10 assay seedlings were planted per treatment.

[f] The first lettuce planting was harvested 7 weeks after infestation with *M. hapla*. A second lettuce crop was planted 2 weeks later and was harvested after 8 weeks.

tation levels has also been reported for *M. javanica* and *H. schachtii* (Lackey et al., 1994; Tedford et al., 1993). Large numbers of *M. hapla* J2 probably escaped infection by *H. rhossiliensis* because of their relatively high density. Also, mobility of J2 probably was greater than the growth rate of *H. rhossiliensis* colonies in soil. Hyphal growth of *H. rhossiliensis* in soil was not measured, but colonies of *H. rhossiliensis* in soil growing out of alginate pellets of *H. rhossiliensis* mycelium reached a diameter of 7.7 mm in loamy sand after 5 weeks (Tedford et al., 1995). Juveniles of *Meloidogyne* spp. can move several centimeters per day (Prot, 1980). Furthermore, frequent high moisture content of the soils in the pots with *H. rhossiliensis,* alone and in combination with *V. chlamydosporium,* probably had a negative effect on spore transmission and sporulation of *H. rhossiliensis*. This phenomenon has also been reported for *H. rhossiliensis* infecting other nematode species (Lackey et al., 1993; Tedford et al., 1992; Timper and Brodie, 1993). *Hirsutella rhossiliensis* could not be detected in the soil after a second lettuce planting, confirming that the fungus is a poor saprophytic competitor and survivor (Jaffee and Zehr, 1985). This was also suggested by the reduced growth rate of *H. rhossiliensis* on nutrient-rich media (1/2 PDA and MA) in the presence of *V. chlamydosporium* compared with the growth rate of *H. rhossiliensis* in pure culture on these media.

Verticillium chlamydosporium became established and survived in the organic soil during the course of all experiments, even when the soil was left unplanted. This was expected because organic soils have been reported to be a better substrate for the growth of *V. chlamydosporium* than mineral soils (de Leij et al., 1993; Kerry and de Leij,

1992; Kerry et al., 1993). Although CFU density cannot be interpreted as a measure of fungal activity or the exact population density in the soil (Kerry et al., 1993), plating of serial soil dilutions on a semi-selective medium allowed for the detection of *V. chlamydosporium* and the comparison of its abundance in different soils that were treated and maintained under the same conditions. The fluctuation in CFU per gram of soil over the course of the experiments can be partially due to the fact that any reproductive unit of the fungus (mycelial fragment, conidium, or chlamydospore) could result in a colony. However, the numbers of CFU per gram of soil clearly increased after the initial application, and the fungus was isolated at every occasion, which indicates that it survived and proliferated in the soil. Proliferation also was demonstrated by the colonization of egg masses at every lettuce harvest.

The percentage of egg masses of *M. hapla* colonized by *V. chlamydosporium* depends on the abundance of egg masses on the root and on the degree of their exposure to the fungus (Bourne et al., 1996; Kerry and Jaffee, 1997). Generally, high infestation levels of *M. hapla* result in more severe galling of lettuce roots and greater production of eggs than low infestation levels (Viaene and Abawi, 1996). The lower percentages of colonized egg masses in soils infested with 8 eggs/cm^3 soil, compared with the percentages of colonized egg masses in soils infested with 2 or 4 eggs/cm^3 soil, can be explained by the larger quantity of egg masses at the higher infestation level, lowering the chance for each egg mass to become colonized, assuming that the abundance of the fungus is the same at all nematode infestation levels. A higher percentage of colonized egg masses also was observed when *M. hapla*-infested soil was diluted with uninfested soil, thus lowering the nematode population density. *Verticillium chlamydosporium* is more effective on less susceptible host plants that produce small galls (Bourne et al., 1996; Kerry and de Leij, 1992). Plants that form large galls at high nematode densities, such as lettuce, are more likely to have most of the egg masses imbedded in the gall tissues. Such egg masses remain protected from colonization by *V. chlamydosporium,* as the fungus cannot penetrate the root cortex (de Leij and Kerry, 1991). In the first experiment, the time between soil infestation with *M. hapla* and inspection of the egg masses for colonization by *V. chlamydosporium* was probably too short (5 weeks) for adequate exposure of the egg masses to the fungus, thus lowering the chances for colonization. However, there was no clear relationship between the severity of root galling and the percentage of egg masses colonized by *V. chlamydosporium* in this study.

Root colonization by *V. chlamydosporium* was also reported to increase its efficacy for parasitizing nematode eggs (Bourne et al., 1996; Kerry and Jaffee, 1997). The numbers of CFU of *V. chlamydosporium* per gram of soil adhering to the roots of lettuce was higher than the numbers in nonrhizosphere soil at only one sampling date. Also, abundance of *V. chlamydosporium* was the same in unplanted soil as in soil planted to lettuce. These results suggest that the rhizosphere of lettuce is not easily colonized by *V. chlamydosporium.* In addition, several attempts failed to isolate *V. chlamydosporium* on a semi-selective medium from 1-cm pieces of lettuce roots grown in *V. chlamydosporium*-treated soil. Only 4 out of 40 root pieces (10%) exhibited colonies of *V. chlamydosporium* in one test, and none were obtained in another. Furthermore, *V. chlamydosporium* was cultured from only 10% of tomato root pieces 3 months after planting in organic soil infested with *M. hapla* and treated with *V. chlamydosporium.* Nonetheless, egg masses on the tomato roots were heavily colonized by the fungus. These results indicate that the lack of root colonization by *V. chlamydosporium* was not due to the assay plant species, since the tomato rhizosphere was well colonized by *V. chlamydosporium* in previous studies (Bourne et al., 1996; de Leij et al., 1993). The root dilution plating technique described by de Leij and Kerry (1991) was equally unsuccessful for detecting *V. chlamydosporium* on lettuce roots. Perhaps the organic soil used in this experiment contained microorganisms that competed with *V. chla-*

mydosporium in the rhizosphere and reduced its establishment. This was also suggested by the bacterial colonies often observed on the root pieces of lettuce and tomato. Further investigation of rhizosphere and root colonization by *V. chlamydosporium* in organic soil from New York is warranted.

Parasitism of *M. hapla* eggs by *V. chlamydosporium* resulted in significant reductions in J2 penetration into roots of lettuce seedlings, as observed after almost every harvest of the experiments with *V. chlamydosporium.* However, suppression of J2 entry into roots of 8 to 14-day-old lettuce seedlings only occasionally resulted in a reduction in *M. hapla* egg production on lettuce roots or in an increase in lettuce weight. It is possible that egg hatch continued for longer than 8 to 14 days, so that large numbers of nematodes eventually infected the roots. Also, although fewer J2 entered the roots of the lettuce plants, their number could have been sufficient to produce as many eggs as the number of J2 entering roots in the control treatments. *V. chlamydosporium* was reported to suppress the population of *M. hapla* on tomato roots by 90% in plastic-covered microplots (de Leij et al., 1993). However, the nematode infestation level was lower (1,000 *M. hapla* J2/plant) than in the present study. Similar results to those of the current study were obtained with *M. arenaria* infecting tomato (de Leij and Kerry, 1991), where *V. chlamydosporium* infected 35% of *M. arenaria* eggs without affecting nematode reproduction.

Verticillium chlamydosporium suppressed populations of the cereal cyst nematode (*Heterodera avenae*) in fields grown to susceptible cereals (Kerry et al., 1982). It was also effective against *M. hapla* and *M. incognita* on tomato in the greenhouse and in field microplots (de Leij et al., 1993). *Hirsutella rhossiliensis* reduced penetration of J2 of *H. schachtii* and *M. javanica* into cabbage and tomato seedlings in pot experiments (Lackey et al., 1994; Tedford et al., 1995). In the present study, application of *V. chlamydosporium* and *H. rhossiliensis,* singly or together, did not result in reduced final population densities of *M. hapla* on lettuce when the initial population density was the damage threshold density (8 eggs/cm^3 soil). However, *V. chlamydosporium* was able to reduce reproduction of *M. hapla* on a second lettuce planting in diluted soil. Application of *H. rhossiliensis* to soil resulted in reduced invasion of lettuce seedlings by J2, which suggests that the fungus is able to control the population of *M. hapla* at low initial nematode infestation levels. This suggests that application of *V. chlamydosporium* and *H. rhossiliensis* in commercial lettuce fields could be effective when low levels of *M. hapla* are present in the soil, or when they are used in combination with other management tools. *Verticillium chlamydosporium* survives well in organic soil, and just a few applications of the fungus might suffice to obtain adequate and lasting control. In contrast, inundative release of *H. rhossiliensis* would be necessary at every lettuce planting, as the fungus did not survive over long periods and is density-dependent. Although the results obtained so far are promising, much more research is needed before these biocontrol agents can be successfully applied to control *M. hapla* under field conditions. Many other factors besides initial nematode population density influence the efficacy of the fungi, the reproduction of *M. hapla,* and plant growth. These factors should also be studied to obtain a better understanding of the interactions between the biocontrol agents, *M. hapla,* and lettuce.

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