European Journal of Plant Pathology **102:** 115–122, 1996.

Suppression of potato cyst nematode root penetration by the endoparasitic nematophagous fungus *Hirsutella rhossiliensis*

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Accepted 28 May 1995

Key words: biological control, *Globodera pallida*, *Hirsutella rhossiliensis*, nematophagous fungi

Abstract

The endoparasitic nematophagous fungus *Hirsutella rhossiliensis* was tested for its ability to suppress root penetration and cyst formation by the potato cyst nematode species *Globodera pallida*. Isolates of *H. rhossiliensis* were obtained from infected potato cyst nematode juveniles from different starch potato fields in The Netherlands. The isolates showed no difference in spore adhesion to juveniles on agar plates (adhesion rate: ± 90%). The most rapid growing isolate, CBS 108.94, was used for experiments. Vegetative mycelial colonies of *H. rhossiliensis* CBS 108.94, grown in potato dextrose broth, were used as soil inoculum. During submerged cultivation the mycelial colonies produced phialides (spore-bearing cells) but no spores. Exposed to the air, however, spores were rapidly formed. The effect of different soil inoculum densities of mycelial colonies on root penetration by *Globodera pallida* was examined in an experiment in 250-ml pots. Up to a mycelial colony concentration representing a potential spore density of 10^4 g^{-1} soil no suppression occurred. At approximated densities of 2.5×10^4 and 10^5 spores g^{-1} soil the numbers of juveniles which penetrated roots were reduced by 30% and 34%, respectively. The distribution of the inoculum could be improved by fragmentation of the mycelial colonies before soil inoculation. Using mycelial fragments, again no suppression of root penetration was observed up to a potential spore density of 10^4 g^{-1} soil, but at densities of 10^5 and 10^6 g^{-1} a suppression of 54% and 88%, respectively, was measured. In a greenhouse experiment, soil inoculation with mycelial colonies with a potential spore production of 2.5×10^5 g^{-1} soil resulted in a suppression of root penetration of 37% and 51% after 5 and 6 weeks, respectively, but the number of newly formed cysts after 18 weeks in soil was not different for control and inoculated pots. It is concluded that *H. rhossiliensis* may be useful for the reduction of root damage caused by juveniles of potato cyst nematodes, but the usefulness for population control is doubtful.

Introduction

Recently, we reported the endoparasitic nematophagous fungus *Hirsutella rhossiliensis* to be the most common fungal parasite of free-living second stage juveniles (J2) of potato cyst nematodes (PCN) in an area of The Netherlands frequently cropped with potatoes grown for the starch industry [Velvis and Kamp, 1995]. In soil samples from 20 fields in that area, up to 40% of the extracted J2 proved to be infected by this fungus. *Hirsutella rhossiliensis* was also found to be an important parasite in field populations of plant pathogenic nematodes, such as *Criconemella xenoplax* and *Heterodera schachtii* [Jaffee and Zehr, 1982; Müller, 1982; Jaffee *et al.*, 1991]. The fungus has a broad host range among plant parasitic nematodes [Nuñez-Fernandez, 1992; Cayrol *et al.*, 1986; Timper and Brodie, 1993], and also seems to be more or less specific to parasitic nematodes [Jaffee *et al.*, 1994]. This makes *H. rhossiliensis* an attractive candidate for biological control.

We performed some preliminary experiments in which we investigated the capability of *H. rhossiliensis* to suppress root penetration by the PCN species *Globodera pallida* during the relatively short period in which hatched J2 move from the cysts to the roots,

and also the approximate density at which suppression may be expected.

1. For that purpose we chose an isolate out of a collection of isolates, recently obtained from field populations of J2 [Velvis and Kamp, 1995], supposedly adapted to the soil environment, possessing the requisites of a good (*in vitro*) spore adhesion, and an optimal activity at different temperatures.
2. Because single spores of *H. rhossiliensis*, detached from phialides (the spore-bearing cells), do not adhere to juveniles [McInnis and Jaffee, 1989; Velvis and Kamp, 1995], we used vegetative mycelial colonies (MC) for soil inoculation, according to Lackey *et al.* [1992].
3. In experiments with 250-ml pots filled with soil and inoculated with different densities of *H. rhossiliensis* (MC), we measured the effect on J2 root penetration, and in a greenhouse experiment with 4-l pots we investigated whether reduction in root penetration of J2 finally leads to a lower number of newly produced cysts in the soil.

Materials and methods

Selection of H. rhossiliensis *isolate*

Five isolates of *H. rhossiliensis* have been deposited in the culture collection of CBS in Baarn, The Netherlands, under CBS numbers 104.94–108.94 [Velvis and Kamp, 1995]. These isolates represented a broader collection of isolates, obtained from different experimental plots and fields in The Netherlands as parasites of PCN juveniles.

Temperature growth-response curves. The temperature growth-responses of the 5 fungal isolates were measured on malt extract agar (MEA) plates (containing 20 g malt extract and 15 g agar l^{-1} water). Circular discs of 2 mm diam., cut out of 10-day old cultures of the isolates on MEA, were placed upside down in the centre of new MEA-plates. The dishes were sealed with Parafilm and placed, in triplicate, at a range of temperatures: 5, 10, 15, 20, 25 and 30 °C. After 35 days the radii of the colonies were measured, starting from the margin of the discs (4 crosswise measurements per plate).

Spore adhesion rates. For the assessment of the spore adhesion rate of the isolates, equal amounts of fragments, obtained from mycelial colonies in liquid culture, were distributed over water agar (WA; 20 g agar l^{-1} water) in 15- cm Petri dishes. The production of the mycelial colonies is described below. Excess water was evaporated in a laminar flow cabinet. The plates were incubated for 24 h at 20 °C. The amount of spores produced by the fragments was approximately 200 mm^{-2}. A suspension with 250 freshly hatched J2 of *Globodera pallida* was pippetted in the centre of each plate, and excess water was evaporated. The plates were again placed at 20 °C. After three days the J2 were rinsed from the plates and 100 individuals were observed for adhering spores with an inverted microscope (Leitz Labovert, magnification 40–250×).

Production of mycelial colonies for soil inoculation

The procedure for the production of vegetative mycelial colonies (MC) was standardized for *H. rhossiliensis* [CBS 108.94]. A spore suspension of this isolate, obtained by suspending spores of cultures on MEA-plates in sterile demineralized water, was filtered aseptically through double layer nylon gauze (Scrynel NY 63 HD-Super) with a pore size diameter of 70 μm. Spores were added to 200-ml Erlenmeyer flasks containing 100 ml potato dextrose broth (PD-broth; Difco) to a density of 6×10^2 spores per ml. The cultures were incubated during 12 days on an orbital shaker (100 rpm) at 20 °C. The MC were collected on a 1-mm sieve and rinsed with 1 l of sterile demineralized water. The number of MC remaining on the 1 mm sieve was approximately 5×10^3 per 100 ml PD-broth. The diameter of the MC in water was between 1.7 and 2.0 mm.

For the detection of the number of spores produced per MC, aliquots of 50 MC in demineralized water were sucked off aseptically on 50-mm diameter membrane filters with a pore size diameter of 1.2 μm, so that the water is drawn from the colonies. The membrane filters were placed in moist-chambers, consisting of 90-mm plastic Petri Dishes with a sterile wet filter paper stuck into the lid and sealed with Parafilm, and incubated at 20 °C. On a number of successive days MC were harvested. MC from each membrane filter were transferred to 25 ml of sterile demineralized water in a centrifugation tube. The MC were fragmented during 3 min with a laboratory mixer-emulsifier [Silverson], and the number of spores in this suspension was counted using a haemacytometer.

Design of pot experiments

Soil used for pot experiments. For the pot experiments a sandy soil was used, sampled from a field in the starch potato cropping region, with an organic matter content of 12.9% and a pH of 6.7. The soil was pasteurized for 2 h at 60 °C on 3 successive days, and reinfected with a bacterial suspension from the same soil. The bacterial suspension was obtained by filtering a soil suspension through a 1.2-μm pore size membrane filter. The reinfected soil was kept, at room temperature, for 2 months before the first pot experiment started.

All pot experiments were performed at a soil matric potential of approximately −100 mbar (= pF 2). Small pots (250 ml) were weighed daily and adjusted to initial weight by water addition, with a correction for the estimated plant weight. Large pots (4 l) were provided with tensiometers and daily supplied with water to the desired water potential.

Suppression of root penetration by juveniles at different densities of H. rhossiliensis. The experiment with different densities of *H. rhossiliensis* was performed in small plastic pots of 250 ml. Pots were inoculated with 0, 15, 150, 375 or 1500 MC, to approximate densities of 0, 10^3, 10^4, 2.5×10^4 and 10^5 spores g^{-1} dry soil. A pre-germinated potato sprout on a small block (15×15×10 mm), cut-out from a tuber, was placed 1 cm below the soil surface. Each pot was infested with cysts of *G. pallida* to an initial density of 10 hatchable eggs g^{-1} dry soil. The pots were placed in a constant environment chamber at 20 °C with a light/dark regime of 16/8 h. The number of juveniles in the roots was assessed (see below) after 4 weeks. This experiment was performed twice with either 3 or 4 replicates per treatment.

The experiment was repeated using mycelial fragments instead of MC as soil inoculum. Fragments were obtained by macerating MC during 30 seconds with a mixer emulsifier. Comparison of the numbers of spores produced by the fragments with those of an equal amount of intact MC showed that there was no decrease by fragmentation. Pots were inoculated with different quantities of fragments to approximate spore densities of 0, 10^3, 10^4, 10^5 and 10^6 g^{-1} dry soil, i.e. 0, 15, 150, 1500 and 15000 fragmented MC per 250 ml soil. This experiment was performed with 4 replicates. The design was the same as in the first experiment.

Greenhouse experiment on suppression of root penetration by juveniles and of cyst formation. In the greenhouse experiment one density of MC was used, 6×10^4 MC per pot of 4 l, based on the information from the first experiment in small pots. The applied density was 2.5× the highest density in the first experiment, with an estimated spore production of 2.5×10^5 g^{-1} dry soil. Soil was again infected with cysts of *G. pallida* to an initial density of 10 hatchable eggs g^{-1} dry soil. A seed tuber (cv. Mentor) was planted in each pot, but also control pots without seed tubers were incubated. The pots were placed in a greenhouse at 18 °C. The number of juveniles in the roots was assessed after 5 and 6 weeks, in triplicate pots. After 15 weeks the foliage was cut-off from the remaining potato plants to enforce cyst maturation, and a mixed soil sample was taken from each pot after 18 weeks (each treatment in triplicate). The soil samples were air-dried, and 100 g of the dried samples was elutriated in a Fenwick can to extract the cysts.

Method of extraction of juveniles from the roots. Juveniles were extracted from the roots by a modification of a maceration-centrifugal-flotation method for recovering of nematodes from plant tissue, as described by s'Jacob and Van Bezooyen [1984]. The roots of the potato plants were washed free from soil during 3 min in running tap water. After removing excess water on filter paper, the roots were weighed, cut into segments of 1 cm length, and fragmented for 18 seconds in 500 ml tap water in a domestic blender. The suspension was rinsed over a set of 500- and 25-μm-mesh sieves. The residue from the 500-μm sieve was again blended for 18 seconds and rinsed over the sieves (3×). The residue from the 25-μm sieve was distributed over five centrifugation tubes of 30 ml. To each tube 0.5 g Kaolin was added, and the tubes were 3/4 filled with tap water. After homogenization on a Vortex for 20 seconds, the suspensions were centrifuged for 5 min at 1800G. The supernatant was discarded and the pellets were resuspended (20 seconds) in a $MgSO_4$ solution with a specific gravity of 1.20 and again centrifuged for 5 min at 1800G. The supernatant was sucked-off on a membrane filter device (Sartorius) with a 8-μm pore size diameter membrane filter. The $MgSO_4$ step was repeated. The fraction on the membrane filter was washed three times with tap water and rinsed into a counting dish. Numbers of nematodes were counted under a stereo microscope (Wild M3).

Differences between means of treatments and control were statistically analyzed by paired testing with Student's t-test.

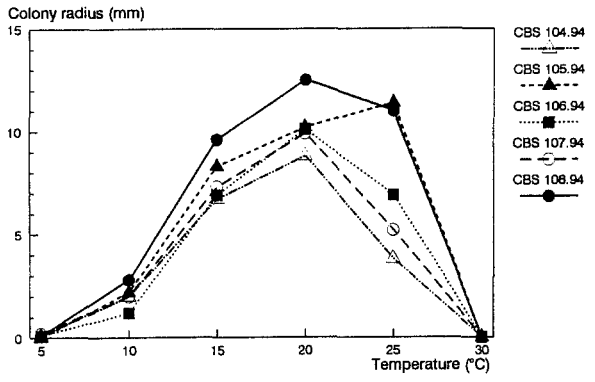

Fig. 1. Temperature-growth-response curves of five *Hirsutella rhossiliensis* isolates from field and plot soils. Colony radii were measured on malt extract agar after 35 days of growth.

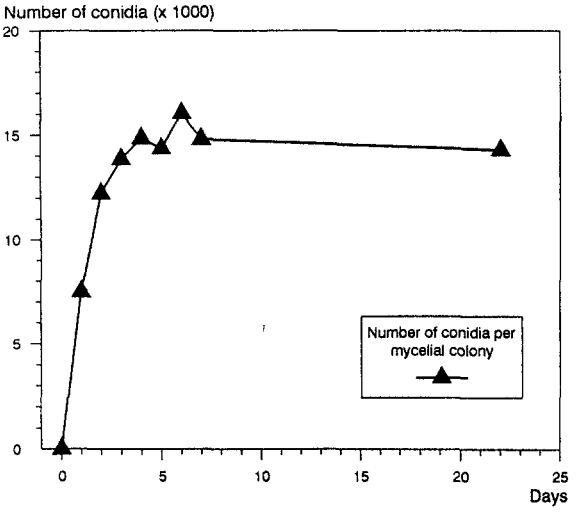

Fig. 2. Production of conidia in mycelial colonies of *Hirsutella rhossiliensis* (CBS 108.94). Each point represents the average of six counts. Standard Error: 2.023.

Results

Growth characteristics of five H. rhossiliensis *isolates and conidial adhesion to J2 of* G. pallida

The optimum temperature for growth on MA was about 20 °C for 4 isolates and 25 °C for 1 isolate (Fig. 1). At 5 °C and 30 °C there was no growth at all. Between 10 and 20 °C the isolates differed moderately in their temperature growth response, at 25 °C the differences were more pronounced. Isolate CBS 108.94 was the most rapid growing isolate over the total range of temperatures.

The percentages of J2 with at least one spore adhering to the cuticle, were 91%, 83%, 86%, 82% and 92% for the isolates CBS 104.94–108.94, respectively. Analyses of variance revealed that the differences between the isolates were not statistically significant ($P > 0.05$). As the *in vitro* adhesion rate was comparable for all isolates, we chose the most rapidly growing isolate CBS 108.94 for further experiments.

The production of spores within mycelial colonies (MC) of H. rhossiliensis *[CBS 108.94]*

The conidial production within MC of isolate CBS 108.94 on membrane filters in moist chambers is plotted against time in Fig. 2. At day zero no spores were present. It was found that in liquid culture no spores were produced, even after prolonged incubation, although there were a lot of phialides present along the hyphae. As soon as the MC were exposed to the air in the moist chambers, spores developed from the phialides, and within 5 days the maximum number of spores (about 1.5×10^4 per MC) was reached. From these data we may conclude that MC of isolate CBS 108.94 meet the requirements of a soil inoculum of *H. rhossiliensis*: no loose spores, which would not adhere to juveniles, but a lot of spore-producing cells, which will potentially produce spores shortly after introduction into soil.

Suppression of root penetration by juveniles at different densities of H. rhossiliensis *[CBS 108.94]*

The two trials with different densities of MC gave comparable numbers of juveniles in roots for each treatment, so the results of both trials were combined (Fig. 3). Average fresh root weight was 2.5 g ± 0.7 g and did not differ significantly among treatments. At MC densities up to 150 MC per pot, with a potential spore production of up to 10^4 g^{-1} soil, root penetration was not reduced. At densities of 375 and 1500 MC per pot, i.e. 2.5×10^4 and 10^5 spores g^{-1}, root penetration was suppressed by 30% and 34%, respectively, as compared to the control ($P < 0.05$ and $P < 0.1$, respectively).

By using MC as inoculum, the distribution through the soil is heterogeneous, because developing spores are confined to the compact mycelial colony. In a similar experiment (personal observation), we obtained 20% more suppression when we used fragmented instead of intact MC. So, we decided to repeat the

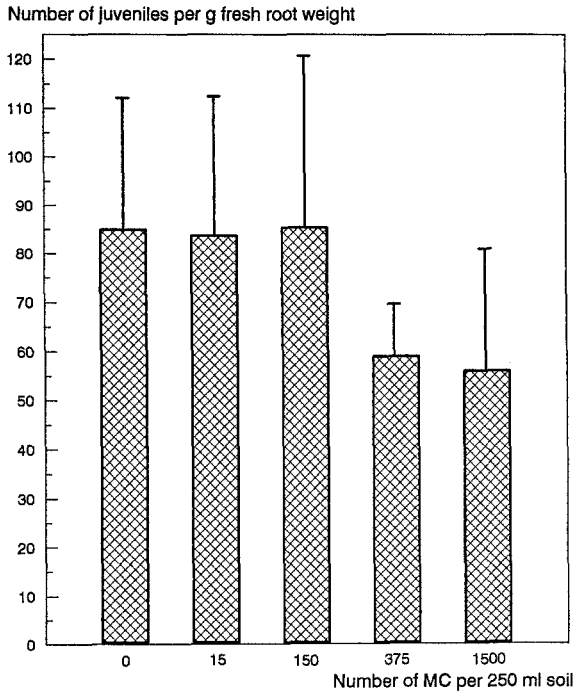

Fig. 3. Effect of *H. rhossiliensis*, added to soil as mycelial colonies (MC), on the penetration of potato roots by juveniles of *G. pallida*. Data from two trials are combined. Fungal concentrations 375 and 1500 MC differed from the control ($P < 0.05$ and $P < 0.1$). Reduction in root penetration: 30% and 34%, respectively.

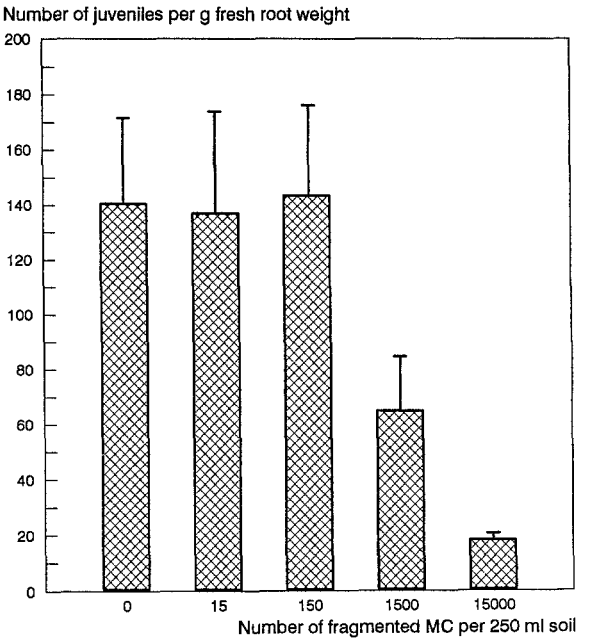

Fig. 4. Effect of *H. rhossiliensis*, added to soil as mycelial fragments, on the penetration of potato roots by juveniles of *G. pallida*. Fungal concentrations 1500 and 15.000 MC differed from the control ($P < 0.01$ and $P < 0.001$). Reduction in root penetration: 54% and 88%, respectively.

experiment with different densities of the fungus by using mycelial fragments as inoculum.

As in the experiment with MC, up to a density of 150 fragmented MC per pot, with an estimated density of 10^4 spores g^{-1} soil, no suppression occurred (Fig. 4). At a density of 1500 fragmented MC, i.e. 10^5 spores g^{-1} soil, root penetration was suppressed by 54%, which was 20% more than the suppression measured in the first experiment at the same density; at a density of 15,000 fragmented MC ($\pm 10^6$ spores g^{-1}) a suppression of 88% was measured. The observed suppression was statistically significant ($P < 0.01$). Average fresh root weight was 2.8 g $\pm$ 0.3 g, without significant differences among treatments.

The effect of H. rhossiliensis *[CBS 108.94] on juvenile root invasion and cyst production (greenhouse experiment)*

The pot experiment in the greenhouse was started before the trials with fragmented mycelial inoculum, so that we still used MC as soil inoculum in this experiment. After 5 weeks (Fig. 5; left part of the diagram) the number of nematodes in the roots was reduced by the fungus by about 37%, but this was not statistically significant as a result of a large variation within treatments. After 6 weeks the variation was less, and then the reduction of 51% was statistically significant ($P < 0.05$). The total numbers of juveniles per g root material decreased between these two observations, as average fresh root weight increased from 17.9 g ($\pm$ 3.1 g) to 33.3 g ($\pm$ 1.7 g), without significant difference between treatments. *Hirsutella rhossiliensis* did not affect the formation of new cysts (right part of the diagram). No difference in number of cyst per 100 g soil between treated and control pots was observed. The number of cysts in the pots with plants was 16× higher than in the pots without plants.

Discussion

Although infection rates by *Hirsutella rhossiliensis* may be rather high in field soils [Müller, 1982; Jaffee *et al.*, 1991; Velvis and Kamp, 1995], the natural population control of nematodes by the fungus is limited [Jaffee *et al.*, 1989; Tedford *et al.*, 1993]. Yet, this

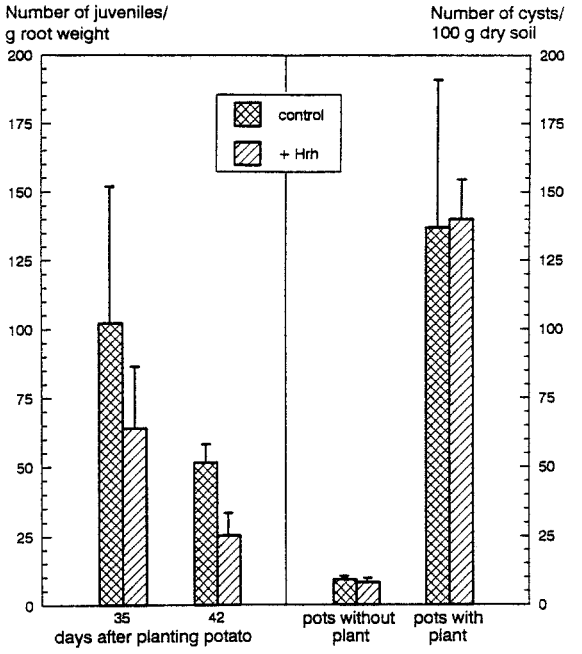

Fig. 5. Effect of *H. rhossiliensis* (Hrh) on juvenile root penetration and formation of new cysts by *G. pallida*.

fungus may be useful for biological control, when additionally introduced to soil at a proper moment and in a sufficient density to suppress root invasion by nematodes.

One of the disadvantages of the use of *H. rhossiliensis* as biological control agent is the phenomenon that single spores of the fungus, detached from phialides, do not adhere to the nematodes [McInnis and Jaffee, 1989; Velvis and Kamp, 1995] and cause infection. Since germination of the spores and subsequent hyphal growth is also suppressed in soil [Jaffee and Zehr, 1985], inoculum for soil or plant treatment should be formulated on a mycelial basis, provided that new infective conidia are formed on the mycelium after introduction into soil. Unlike the *H. rhossiliensis* isolate (IMI 265748) used by Jaffee *et al.* [1992], the isolate we obtained from infected potato cyst nematode juveniles (CBS 108.94) produced large numbers of phialides during submerged growth in potato dextrose broth, although no spores were formed. Lackey *et al.* [1992], also using isolate IMI 265748, reported that the first phialides and spores were observed within two days after incubation in moist chambers, and that radial growth of the mycelial colonies and sporulation continued for at least 3 weeks. Radial growth of mycelial colonies of our isolate CBS 108.94 in moist chambers was very restricted and within 5 days the spore production reached its maximum. So, different isolates of *H. rhossiliensis* may differ in this respect. In both cases, however, the isolates fulfilled the requirements for a suitable soil inoculum with *H. rhossiliensis*.

The suppression of potato root invasion by juveniles of potato cyst nematodes, moving from cysts to roots, requires rather high densities of spores. At levels up to 10^4 conidia g^{-1} soil we measured no reduction in numbers of penetrated juveniles. The conidial numbers are estimated from *in vitro* spore counts from known amounts of MC. The actual production of conidia in soil will be smaller than in the moist chambers, because mycelial inoculum, submerged in water-filled soil pores, will not produce spores [Jaffee *et al.*, 1992]. More than 10^4 conidia, potentially produced by 6×10^2 MC l^{-1} soil, should be added per g soil to have a suppressive effect. To assure a result of about 50% suppression, a density of 10^5 spores g^{-1} (i.e. 6×10^3 MC l^{-1} soil) is recommended. Furthermore, an optimal distribution of the inoculum through the soil is important, which was improved by fragmentation of the MC. The penetration of roots by juveniles could be reduced by almost 90% at an estimated fungal density of 10^6 spores g^{-1}, using fragmented MC, but the amount of mycelium which had to be added to establish this density has to be considered as unpractical (the equivalent of 6×10^4 MC l^{-1} soil).

Suppression of root penetration by plant-parasitic nematodes with *H. rhossiliensis* was also reported by other authors. Lackey *et al.* [1992] obtained 50% suppression of root penetration in cabbage by juveniles of *Heterodera schachtii* after addition of approximately 4 MC of *H. rhossiliensis* per 17 cm^3 soil (= 2.3×10^2 l^{-1}). The colonies were of approximately the same size (1.7 mm) as those used in this study. More recently, Lackey *et al.* [1994] reported 42% and 98% suppression of tomato root invasion in soil infested with *Meloidogyne javanica* egg-masses or juveniles, respectively, and 83% and 98% suppression of cabbage root invasion in soil infested with cysts or juveniles of *H. schachtii*, respectively. They used an inoculum of *H. rhossiliensis* hyphal fragments embedded in alginate pellets, added to soil at a density of 50 pellets per 100 cm^3. The pellets contained an average amount of hyphae equivalent to 1.75 MC and the amount of MC mixed through soil was 8.75×10^2 l^{-1}. The suppression of root penetration by juveniles of potato cyst nematodes probably requires higher densities than beet-cyst and root-knot nematodes. However, the number of spores per unit of mycelium may of course also differ between the isolates used.

For our experiments a soil type was used which is representative for the starch potato cropping area in northern Netherlands, where potato cyst nematodes are a serious problem. The moisture level chosen (matric potential −100 mbar) was considered to be realistic for the field situation in spring, when potatoes are planted. As transmission of *H. rhossiliensis* spores to nematodes may vary with soil type, moisture conditions and nematode species [Tedford *et al.*, 1992], the observed effects of soil inoculation cannot be generalized for different situations or regions.

Although a substantial reduction of root penetration by potato cyst nematodes is possible, it would be worthwhile to select more effective isolates of *H. rhossiliensis*, especially those which infect juveniles at soil temperatures (5–10 °C) typical in spring. For that purpose fungal behavior should not only be considered *in vitro*, but also in non-sterilized soils under field circumstances.

The use of a hyphal suspension is not very convenient for soil inoculation. Pelletizing the hyphal fragments seems to be a good alternative but, using alginate pellets, the inoculum is still concentrated in discrete units of almost the same size as the mycelial colonies. So, efforts have to be made to optimize the formulation and distribution through the soil.

Cyst formation was not reduced after a decrease in root invasion of about 50%. There are some reports of a reduction in cyst formation or multiplication of the beet cyst nematode *Heterodera schachtii* by the fungus *Hirsutella heteroderae* [Juhl, 1985; Müller, 1985], which is considered to be synonymous to *H. rhossiliensis*. Because the number of maturing females on the roots is limited by the carrying capacity of the roots, only a reduction of juveniles in roots below that critical level will lead to effects on the multiplication rate of the nematode. Obviously that limit has not been reached in the greenhouse experiment. The value of *H. rhossiliensis* has to be sought mainly in a reduction of early root damage, and consequently in a reduction of yield loss. Yield reduction is mainly caused by early root infection by the nematodes, so if the plant could be protected during this stage by soil inoculation with *H. rhossiliensis* and/or other organisms, it might be of economic benefit to commercial starch potato cropping. A combination of parasites of second stage juveniles and of developing females on the roots [Crump and Irving, 1992; De Haan and Thijssen, 1991] may be useful in the future in reducing both root damage and nematode reproduction.

As far as *H. rhossiliensis* against potato cyst nematodes is concerned, research has to be done on the selection of more effective isolates, improvement of inoculum formulation and optimization of the distribution through the soil, and the effect of the fungus under field conditions and at different inoculum levels of the nematode. Besides the effects on potato cyst nematodes, *H. rhossiliensis* may also suppress damage by other parasitic nematodes which are resident in the starch potato region.

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