Techniques to determine the activity of fungal egg parasites of *Heterodera schachtii* in field soil

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SUMMARY

Existing methods used to determine fungal egg parasitism of *Heterodera schachtii* populations are unsatisfactory and so two techniques were developed for determination of the activity of egg parasitic fungi in field soils. They are based on the examination of eggs newly formed either in observation chambers or in a soil fraction. The recommended method is : *1)* extraction of all cysts from a soil sample, *2)* release of the eggs and fungi from the cysts by crushing, *3)* reincorporation of the cyst contents into the original soil sample, and *4)* estimation of parasitic activity on newly formed eggs. The proportion of parasitized eggs based on the total number of newly formed eggs reflects the parasitic activity in any particular soil sample. Experiments demonstrated that the rate of parasitism of newly produced *H. schachtii* eggs was not correlated with the nematode density, but increased with increasing fungus density.

RÉSUMÉ

Techniques permettant de déterminer l'activité des champignons parasitant les œufs d'Heterodera schachtii *au champ*

Les techniques actuellement utilisées pour déterminer le parasitisme des champignons envers les œufs d'*Heterodera schachtii* ne sont pas satisfaisantes; aussi, deux techniques nouvelles ont-elles été mises au point pour la détermination de l'activité des champignons parasitant ces œufs, au champ. Elles sont fondées sur l'examen des œufs néoformés, soit en chambres d'observation, soit dans une fraction du sol. La méthode recommandée consiste en : *1)* l'extraction de tous les kystes d'un échantillon de sol; *2)* l'extraction des œufs et des champignons hors des kystes, par écrasement; *3)* l'incorporation du contenu des kystes dans l'échantillon du sol original, et *4)* l'estimation de l'activité parasitaire envers les œufs néoformés. Le nombre d'œufs parasités par rapport au nombre total d'œufs néoformés reflète l'activité parasitaire, et ce pour n'importe quel échantillon de sol. Il a été démontré expérimentalement que le taux de parasitisme envers les œufs néoformés d'*H. schachtii* n'est pas lié à la densité de la population du nématode, mais croît en fonction de l'augmentation de la densité du champignon.

Fungal parasites of *Heterodera schachtii* are important in bioregulation of nematode population densities in all stages of nematode development occuring outside the root. The nematode is susceptible to fungal parasites while in the egg, a stage that may survive several years under nonhost crops. Formalin (Kerry, Crump & Mullen, 1980, 1982) or Captafol (Kerry, 1984) have been used to suppress or prevent fungal parasitism to determine the influence of fungi on population development of cyst nematodes. Increases in nematode density following fungicide treatment have been shown to be partially related to improved root growth. This effect can lead to overestimates of fungal parasitic activity.

The level of egg parasitism in cysts taken from soil following fumigation with Telone or Shell DD or Captafol treatment was not different from the control (Müller, 1983; Weischer & Müller, 1985; Crump & Kerry, 1987). The results demonstrated that this form of treatment is not suitable for determination of fungal parasitic activity in eggs.

In addition to parasitized and nonparasitized eggs, cysts from earlier generations contain large numbers of empty egg shells that cannot be used to calculate fungal parasitism. For example, where large numbers of hatched larvae leave many empty egg shells, investigations based on the level of parasitism in egg suspensions could lead to over-estimations (Lopez-Llorca & Duncan, 1986).

In order to determine the antagonistic potential of fungal parasites of eggs the soil to be analysed must be freed of cysts and the fungi challenged with newly formed eggs. The proportion of parasitized eggs based on the total number of newly formed eggs serves as a measure of the egg parasitic potential of a particular soil.

This research describes two techniques which exclude

old cysts from the evaluation process and base the degrees of parasitism exclusively on newly produced eggs.

Material and methods

General techniques

Assessment of nematode population density in the field

Sixteen soil cores were taken from each 2 × 4 m plot by means of an Oakfield soil sampler (25 cm deep, 2 cm diam.) and the total sample thoroughly mixed. Cysts were extracted from two 250 g subsamples from each plot with a MEKU high pressure elutriator (MEKU, Wennigsen/Deister, FRG). The cysts collected on a 250 µm-sieve were separated from organic matter with a $MgSO_4$ solution (1.28 g/ml) and the number of cysts, eggs and larvae determined.

Evaluation of egg parasitism by fungi

The level of egg parasitism was determined using the following modification of the technique described by Kerry and Crump (1977). The egg suspension was shaken in a test tube to break up clumps of eggs and then poured onto a 20 µm-sieve. The residue of the sieve was washed with deionized water, placed in graduated 15 ml-centrifuge tubes and centrifuged for 5 min at 2 500 rpm in a clinical centrifuge.

The supernatant was removed with a pipette until 0.3 ml remained. A 0.3 ml solution containing 100 mg/l streptomycin sulphate and 200 mg/l penicillin G was added to the 0.3 ml sediment in each tube. The resulting 0.6 ml suspension was spread over a 1.5 % water agar petri dish (9 cm diam.). Large cyst wall fragments were left in the tube. Approximately 1 000 eggs and larvae were applied per plate and the plates incubated at room temperature (ca. 20°). After 48 hr 100 eggs and larvae per plate were randomly scored and the proportion exhibiting fungal growth determined under a stereomicroscope at × 100 magnification.

Observation chambers

The observation chambers were constructed from 9 cm diameter petri dishes (Crump & Kerry, 1977). The lid and the base were held together by a rubber band and the dish was filled with the soil to be examined. A hole was made in the dish bottom to allow free drainage.

The soil samples for this experiment were taken from 30 plots with different population densities in the spring of 1986 before sugar-beet planting. The population density was determined as described above and ranged from 7 to 55 cysts or 753 to 3 045 eggs and larvae per 100 g soil.

Five chambers per soil sample were each filled with 70 g soil having a moisture content of 17 %. Five chambers contained heat-sterilized field soil.

Six seeds of rape *Brassica napus* cv. Akela were seeded per chamber and the chambers placed next to each other at a 45° angle with the lid facing downward in plastic trays filled to a 3 cm depth with moistened sand. The trays with the chambers were incubated at 21° with 16 h artificial lighting (4 000 Lux) in a growth chamber and watered by moistening the sand with deionized water. Each chamber was inoculated with 1 500 *H. schachtii* larvae ten days after seeding.

The experiment was evaluated 40 days after inoculation.

Twenty newly formed cysts were removed from the roots in each chamber after lifting off the lid.

The cysts were crushed in a tissue homogenizer and the rate of egg parasitism determined a described above.

Soil-fractioning technique

Soil samples were taken from 75 field plots with different *H. schachtii* population densities in August 1986 after winter barley. The population density ranged from 9 to 39 cysts or 240 to 1 575 eggs and larvae per 100 g soil.

A 70 g soil sample was suspended in 100 ml water with a vibromixer, poured onto a 250 µm-sieve and washed with water. The fungi in the cysts, parasitized and healthy eggs were freed from the cysts by gently crushing the cysts on the sieve with a rubber stopper. The freed cyst contents were then washed through the sieve with water.

The filtrate containing the eggs as well as fungi was added to 800 ml beakers and centrifuged at 5 600 g for 20 min.

The sediment was mixed 1 : 1.5 (w/w) with sterilized sand to improve its texture and the resulting mixture of each sample added to a 7 cm diam. plastic pot. Eight pots containing heat-sterilized field soil mixed 1 : 1 (w/w) with sand served as controls.

The pots were placed in plastic trays on a 3 cm deep layer of moist sand and ten rape seeds cv. Akela seeded into each pot. The experiment was conducted at 21° in a growth chamber with 16 h artificial lighting (4 000 Lux). The pots were watered with deionized water.

Each pot received 1 000 *H. schachtii* larvae in addition to the natural infestation in the filtrate ten days after seeding. Two pots were prepared for each plot with half of them evaluated 40 and the other half 68 days after larval inoculation.

The seedlings were severed at the soil surface 40 days after inoculation in order to reduce development and hatching of the second generation in the block to be evaluated after 68 days. These pots were stored for the remaining 28 days at 17° to allow further egg parasitism.

The newly formed cysts were extracted and the rate of parasitism determined as described.

EPIDEMIOLOGY OF FUNGAL EGG PARASITISM

An attempt was made to determine the optimum evaluation time for fungal egg parasitism in the soil-fractioning technique. We considered this point to have been reached when a high rate of parasitism occurs at a time when there are few empty eggs or when small differences between empty egg shells and free larvae are detected on the agar plates.

A soil fraction produced using the technique already described was placed into 42 pots (7 cm diam.). The pots were seeded with rape, inoculated ten days later with 1 000 *H. schachtii* larvae/pot, and maintained at 21° in a growth chamber with 16 h artificial lighting (4 000 Lux).

The newly formed cysts in six replicates were extracted at 4, 5, 6, 8, 10, 12 and 14 week intervals after nematode inoculation. The shoots of the plants were removed 40 days after inoculation in the six to fourteen weeks treatments and the pots were stored at 17° until evaluation.

The cysts were extracted and their contents applied to water agar plates as described. The rate of parasitism as well as the proportion of empty egg-shells and free second-stage larvae on the agar plates were determined after 48 h.

The experiment was performed twice.

INFLUENCE OF FUNGAL PARASITE DENSITY ON THE RATE OF EGG PARASITISM

The soil-fractioning technique was used to determine if increasing fungal parasite density was correlated with the level of egg parasitism in *H. schachtii.*

Field soil was treated using the soil-fractioning technique and the resulting soil fraction mixed 1 : 1.5 with sand. A similar soil fraction was sterilized with heat and both soils stored at room temperature for two weeks.

The heat sterilized fraction was mixed with increasing amounts of the nonsterilized fraction until the following treatments were established : 0, 25, 50, 75 and 100 % nonsterilized field soil. Six 7 cm diam. pots were filled with each soil mixture and treated as described above (soil-fractioning technique). The experiment was performed twice.

Results

OBSERVATION CHAMBERS

Observation of the root system through the wall of the chamber was initially easy but became difficult due to the silty soil. This reduced detection of browning cysts on the root surface sometimes to a point at which twenty cysts could not be collected. In these instances the root system was removed from the chamber, washed and the required number of cysts collected. Cysts of all colour stages from white to brown were collected from the roots. There was no correlation between colour of cysts on the roots (percentage brown cysts) and level of fungal egg parasitism (r = 0.21 n.s.).

The rate of parasitism of the newly formed eggs in the observation chambers was used as a measure of the parasitic activity of soil fungi in the soil samples. The mean rate of parasitism was 5.5 %, egg parasitism in the control chambers containing sterile soil was 0.25 %.

The results showed that the rate of parasitism was not correlated to *H. schachtii* egg and larval density in 100 g soil (Tab. 1). There was no statistical evidence of a correlation between the rate of parasitism of newly formed eggs and the number of cysts in the field.

Table 1

Correlation (r-values) between the rate of parasitism of newly formed eggs of *H. schachtii* and the population density in the field using different examination techniques.

Technique	*Eggs and larvae in the field*	*Cysts in the field*
Observation chambers*	0.02 n.s.	0.34 n.s.
Soil-fractioning**		
40 days	— 0.05 n.s.	— 0.07 n.s.
68 days after nematode inoculation	0.11 n.s.	0.10 n.s.

* r-values from 30 observations.
** r-values from 75 observations.

SOIL-FRACTIONING TECHNIQUE

The mean rate of parasitism of newly formed eggs in the block examined 40 days after nematode inoculation was 7.6 % with 0 % in the sterile soil controls.

This rate of parasitism was not correlated with nematode population density in the field (Tab. 1). There was also no correlation between rate of egg parasitism in the pots and number of cysts in the field sample.

The mean rate of parasitism 68 days after nematode inoculation was 7.1 % (control 1 %) and was not significantly different from the 40 day treatment although the cysts were incubated an additional four weeks. Again there was no correlation between the rate of parasitized eggs in the pots and the number of eggs and larvae as well as cysts in the field (Tab. 1). Fungal egg parasitic activity was independent of nematode population density in the range of 240 to 1 575 eggs and larvae per 100 g soil.

A main prerequisite for the practical application of this technique for analysis of field samples for fungal parasitic potential is the absence of a correlation between the rate of parasitism and the number of newly formed

eggs or cysts in the pots. These conditions were met in both the 40 and 68 day evaluation periods (Tab. 2).

Table 2

Correlation (r-values*) between the rate of parasitism of newly formed eggs of *H. schachtii* and the numbers of eggs and cysts/pot 40 and 68 days after nematode inoculation in field soil.

Days after inoculation	*Eggs/pot*	*Cysts/pot*
40	— 0.15 n.s.	0.04 n.s.
68	— 0.20 n.s.	— 0.12 n.s.

* r-values from 75 observations.

EPIDEMIOLOGY OF FUNGAL EGG PARASITISM

The results in Figure 1 show that the rate of parasitism of newly produced eggs was highest ten weeks after nematode inoculation. At this point in time the technique exhibited its greatest level of sensitivity. Differences between different soil samples were most obvious at this

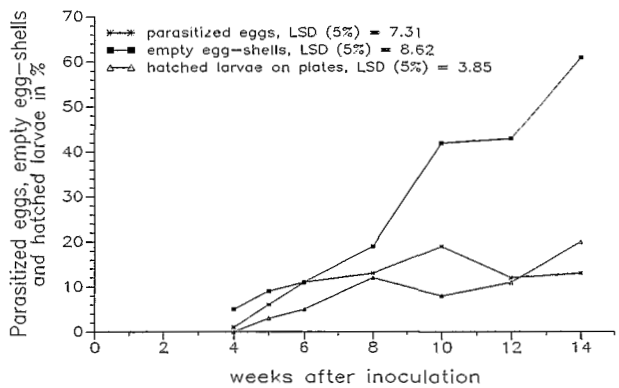

Fig. 1. Percentage of parasitized *Heterodera schachtii* eggs, empty egg-shells and hatched larvae on plates at different evaluation dates. (Means of twelve replicates from two separate experiments.)

time. It should be stressed that empty egg-shells, that do not represent hatched larvae observed on the plates are not considered when calculating the rate of parastism. Evaluation eight weeks after nematode inoculation is considered as optimum because the proportion of empty egg-shells and the difference between them and the number of second stage larvae on the plates increased greatly after ten weeks.

INFLUENCE OF FUNGAL PARASITE DENSITY ON THE RATE OF EGG PARASITISM

The rate of parasitism in newly formed eggs increased with the proportion of unsterilized field soil (Fig. 2). The mean values of twelve replicates show a highly significant correlation between the percentage of unsterile field soil and the percentage of infected eggs ($P < 0.01$).

The results show that the soil-fractioning technique is efficient in detecting different densities and activity of parasitic fungi attacking eggs in field soil.

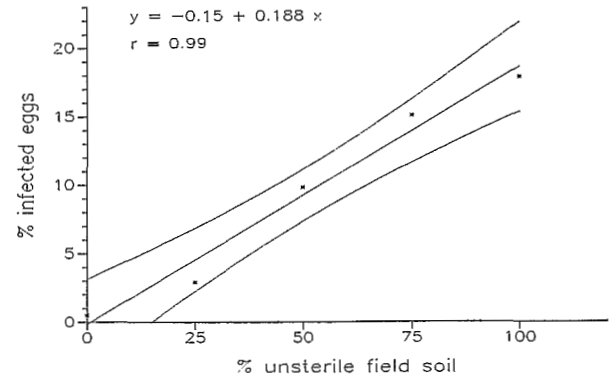

Fig. 2. Correlation between the percentage of unsterile field soil and the percentage of fungus infected *Heterodera schachtii* eggs. (Means of twelve replicates from two separate experiments.)

Discussion

There are five phases in the life cycle of a cyst nematode that can be attacked by parasitic fungi : *(1)* young developing females on the root, *(2)* males, *(3)* embryonated eggs in cysts, *(4)* 1st or 2nd stage juveniles in eggs, and *(5)* active second stage larvae in the soil. Tribe (1979) suggested those parasites affecting the females and cysts on the root and those parasitizing the cyst contents in the soil as being important in the regulation of nematode populations.

Whereas the observation of parasitic activity on eggs in cysts over extended periods of time (years) is problematical, the influence of biotic or abiotic factors on fungal egg parasites can be tested at the time of egg development. With our techniques this may be done at any chosen soil sampling time. The method can be used to compare the effect of different treatments on the level of parasitic activity.

In our studies two techniques were used to determine the influence of *H. schachtii* population density on the activity of fungal egg parasites. Both techniques were based on the assumption that high levels of hyphae or spores of egg parasitic fungi in field soil will be reflected in high rates of egg parasitism when the fungi are challenged with newly forming eggs in young females.

Since egg parasites may also attack females before or during egg production, it is advisable to make an additional estimation of female parasitism or to determine the number of newly formed cysts and cyst contents.

This information would give a more comprehensive view of overall parasitic activity.

A prerequisite for exact measurement is the removal of all cysts from the field sample (on the other hand not their contents) to exclude examination of parasitized eggs in older cysts that can negatively influence the results.

The study of only newly formed eggs has the advantage that few eggs are empty due to previous hatching activity. The fact that egg parasitic fungi usually leave a parasitized egg after the nutrients are utilized leaving an empty egg-shell prevents determination as to whether the empty egg is the result of parasitism, physiological death or the hatching process (Fig. 3).

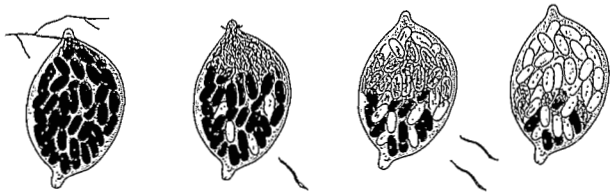

Fig. 3. Theoretical epidemiology of fungal egg-parasitism within a cyst.

Stirling (1979) stated that the incubation of newly forming eggs in soil is appropriate to detect egg parasitic fungi because fungi that attack young eggs are probably parasitic rather than saprophytic on nematode eggs.

The observation chambers made by Crump and Kerry (1977) were originally used to observe the degradation of newly formed females by parasitic fungi. In our studies they were used to aid in recovery and separation of newly produced cysts from older cysts already present in the soil to be examined. The buildup of a muddy silt film on the chamber surface prevented clear examination of the root surface. Disloging of cysts from the roots further hampered the efficiency of this chamber for work with some types of field soil.

The soil-fractioning technique developed here removes old cysts through sieving and allows reintroduction of the fungi and eggs inside the cysts following crushing. It allows the use of all the cysts that developed on the roots and not just a subsample for quantitative collection of data on the parasitic potential in a field soil.

Our results showed that the activity of egg parasitic fungi is not correlated with the nematode density in the field at population densities usually occuring under practical conditions. The rate of parasitism of newly formed eggs was not influenced by the number of eggs and larvae per 100 g field soil nor by the number of cysts. The results indicate that the cyst does not act as the main site of fungus multiplication. It may be assumed, however, that cysts can serve as survival agents as well as propagation units for egg parasites.

Our ability to detect increasing rates of parasitism with increasing fungal density demonstrates the applicability of the soil-fractioning technique for examination of fungal egg parasitic activity in soil. The fact that parasitism was decreased with decreasing spore levels showed that the spread of egg parasitic fungi in soil is highly restricted.

The study of the epidemiology of fungal egg parasitism resulted in the establishment of the optimum time for evaluation at eight weeks after nematode inoculation on rape using the soil-fractioning technique. The reason for the lack of an increase in the rate of parasitism between 40 and 68 days after inoculation is not known. However this result does not prevent an evaluation after 54 days because the difference in the numbers of empty egg-shells and second stage larvae on the plates remained small at this point of time. Later experiments showed that with this technique infection rates of more than 90 % may occur indicating that parasitic activity is not hindered by the disturbance or dilution of the soil.

The level of parasitism did not increase beyond ten weeks after inoculation indicating fungal consumption of the contents of many eggs leaving empty egg-shells.

A slight increase in the proportion of eggs infected with fungi during population developement found by Crump and Kerry (1987) was considered the result of larval hatch from healthy eggs. Both phenomena demonstrate the deficiencies of direct examinations based on the rates of parasitism of cysts extracted from field soil.

Rape was utilized in our studies because fungicide treated sugarbeet seed affects the rate of parasitism and untreated seed did not survive.

The soil-fractioning technique allows fungal parasites attack of all development stages from egg production to second stage larvae formation. The direct microscopic detection of parasitism in newly produced eggs circumvents problems encountered in studies using fumigants or fungicides to estimate parasitic activity. Although highly accurate quantitative prediction of the influence of fungal egg parasitism on the population dynamics of *H. schachtii* in the field is not possible, our technique allows comparisons between different fields and different treatments. The technique is applicable for studies concerning the influence of agronomic and environmental factors on the activity of fungal egg parasites of *H. schachtii* and possibly other cyst nematodes in soils from different fields with similar texture.

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