*Fundam. appl. Nematol.*, 1992, 15 (5), 449-455

# Persistance and growth of an egg pathogenic fungus applied in alginate granules to field soil and its pathogenicity toward *Globodera pallida*

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Accepted for publication 4 December 1991.

**Summary** — The effect of a fungal pathogen incorporated into alginate granules on *Globodera pallida* population dynamics was investigated and the effects found were due to reduced root weight up to 76.4 % after 2 weeks, which led to similar reductions in hatching and in root penetration. Comparison between the number of newly formed cysts and level of infected *G. pallida* eggs from different own experiments showed a clear negative correlation. A closer examination proved, that only inoculated cysts were infected and not newly formed ones. Even so the fungus was able to infect newly formed females; an experiment concerning the duration of infectivity in field soil proved a decrease in the level of infection of eggs 28 days after application of the fungus. Duration of fungal infectivity was examined 2 and 8 weeks after granule application. There were no differences in degree of infection but a reduction in the number of eggs per cyst. Fungal growth through field soil was measured with a template system using the degree of infection of *G. pallida* eggs as a parameter which showed high levels of infection when cysts were directly attached to the fungal inoculum. Perthophytic mode-of-action of the isolate used is discussed.

**Résumé** — ***Persistance et croissance d'un champignon parasite des œufs appliqué en champ sous forme de granulés d'alginate; sa pathogénie envers* Globodera pallida** — L'influence d'un champignon pathogène incorporé à des granules d'alginate sur la dynamique des populations de *Globodera pallida* a été étudiée. Les effets observés consistent en une diminution du poids des racines pouvant aller jusqu'à 76,4 % après deux semaines, ce qui induit une diminution de même ordre pour l'éclosion et la pénétration du nématode. Lors de ces expériences, la comparaison entre le nombre de kystes néoformés et le taux d'infestation des œufs de *G. pallida* relève d'une corrélation négative nette. Un examen plus attentif montre que seuls les kystes inoculés sont infestés et non les kystes néoformés. Même dans ces conditions, le champignon peut infester les femelles néoformées; une expérience en champ sur la persistance du pouvoir infestant du champignon montre une diminution du taux d'infestation des œufs, vingt-huit jours après l'application du champignon. La persistance du pouvoir infestant du champignon a été testée deux et huit semaines après l'application des granulés : il n'y a pas différence dans le degré d'infestation, mais une diminution du nombre des œufs par kyste. La croissance du champignon en champ a été mesurée grâce à un système calibré utilisant le degré d'infestation de *G. pallida* comme paramètre, lequel démontre de hauts niveaux d'infestation lorsque les kystes sont directement au contact de l'inoculum fongique. Le mode d'action perthophytique de l'isolat utilisé est discuté.

**Key-words :** Nematodes, *Globodera*, pathogenic fungus, population dynamics, alginate.

A broad spectrum of fungi have been shown to have the ability to infect the eggs of cyst nematodes (Kerry, 1974; Goswami & Rumpenhorst, 1978, Nigh *et al.*, 1980; Roessner, 1987). However only a few studies (Dackmann, 1990) have been conducted with *Globodera pallida*, these fungi are potential biological control agents especially in those situations where resistant cultivars are lacking or in water conservation zones, where nematicide use is prohibited. The lack of commercially acceptable application technology for fungal based biological control systems has limited their introduction into commercial agriculture. For example, the application of fungal egg pathogens for *G. pallida* as; spore or mycelium suspensions, on colonized straw or bran proved to be unsuccessful (Sikora *et al.*, 1990). Successful formulation of fungi for the biological control of fungal plant pathogens has been accomplished by incorporating the fungal agent into alginate granules; a technique first used by Walker and Connick (1983) for mycoherbicides. Schuster and Sikora (1992) demonstrated that perthophytic fungal egg pathogens of *G. pallida* could be incorporated successfully into field soil when formulated in alginate granules. The technique increased biological control efficacy significantly under field conditions. To maximize efficacy of the alginate formulation system however, more detailed studies are needed on the interrelationship between the nematode, fungi and alginate in the soil ecosystem. The following goals were set to gain a better understanding of this interrelationship : *i)* effect of fungal pathogens incorpor-

*ISSN 1164-5571/92/05/449 07 $ 2.70 / © Gauthier-Villars - ORSTOM*

ated into alginate granules on nematode population dynamics; *ii)* influence of alginate formulation on survival and growth of fungal pathogens in field soil, and *iii)* determination of optimum conditions for fungal infection of eggs.

## Material and methods

### General methods

*Isolate and cultivation of the fungus*

A non-sporulating fungus (Fs), originally isolated from *G. pallida* eggs and initially shown to cause high levels of infection of *G. pallida* eggs in *in vitro* tests (Sikora *et al.*, 1990), was used in all experiments. The isolate caused high levels of infection when applied in alginate granules to field soil in pot experiments. The isolate was considered to be a perthophytic pathogen killing eggs before colonization by the action of toxins (Schuster & Sikora, 1992).

Inoculum was produced by : 1) growing the isolate on 1 % potato dextrose agar (PDA) in Petri dishes at 25 °C for 5 days, 2) inoculating individual 1 litre screw cap culturing flasks containing 500 ml of a glycerine-peptone nutrient broth with the fungal biomass and agar of one Petri dish, and 3) incubating the cultures on a rotary shaker for 7 days at 21 °C. Fermentation was terminated when approximately 40 g fresh weight (0.85 g dry weight) of fungal biomass was present in the flask.

*Production of alginate granules*

The fungus was incorporated into alginate granules as described by Schuster and Sikora (1992). In all but one experiment, the granules contained the culture media glycerine-peptone broth plus 2 % bran. Actual fungal biomass in the granules was approximately 0.005 g fresh weight/granule. An application rate of 1 % (w/w) consisted of 330 granules/100 g soil equivalent to 1.7 g fungal fresh weight/100 g soil.

The following nutrient combinations were used in the alginate granules in the test to determine the effect of nutrient level on biological control : 1) ABNF = alginate + culture media + wheat bran + Fs; 2) ABN = alginate + culture media + wheat bran; 3) AF = alginate + Fs; 4) A = alginate.

The fungus was separated from the broth in the granules without nutrients (AF), using a 100 µm sieve and the fungus resuspended in distilled water.

Fungal density per unit soil after treatment was not measured, because fungal growth out of the granules as mycelium is a dynamic process varying quickly within a short period of time. This makes quantification of a specific fungus in non-sterile field soil, without specific marking techniques or a selective, nearly impossible.

*Evaluation*

Cysts were separated from the soil using the wet screen-decantation method (modified from Ayoub, 1977) and the cysts separated from the remaining organic matter by flotation in $MgSO_4$ at a density of 1.285 g/ml.

Biological control was determined by estimating fungal infection levels of eggs following crushing of the cysts in a hand-held tissue homogenizer. The eggs from the crushed cysts were collected on a 25 µm sieve and the resulting egg suspension plated onto agar plates. The degree of infection was determined using the technique described by Schuster and Sikora (1992).

The soil used was a mixture of cyst free field soil and sand (1:1). The experiments were conducted in either the greenhouse or in climatic chambers at a mean temperature of 21 °C with 16 h illumination.

### Experimental design

*Effect of fungal formulation in alginate granules on potato root weight, nematode emergence and penetration*

*G. pallida* soil from greenhouse stock cultures was added to a field soil : sand-mixture (1:1) until a pi of 1500 eggs and juveniles in cysts per 100 g of soil was obtained. Greenhouse plastic pots 11 cm in diameter were then filled with 600 g of this soil mixture. Each treatment was replicated eight times. Alginate granules (ABNF; ABN; AF; A) were incorporated into the soil at 1 % (w/w) prior to planting by thoroughly mixing the total amount of soil required for each treatment with the granules before potting (4.8 kg soil : 48 g alginate granules). The nematode infested controls were not treated with granules. One certified seed tuber of the cultivar " Hansa ", 3.5 cm in diameter, was planted into each pot. Fresh root weight was determined 2 or 8 weeks after planting.

The effect of the Fs-alginate formulation on hatch was determined two weeks after planting. The pots were flooded two times within 3 h with 150 ml distilled water and the solution collected from below the drainage hole in the pots. The solution was then pre-filtered on a cottonwool filter (Schleicher & Schuell Nr 311607) and passed through a 0.2 µm filter (Sartorius SM 11307) in a sterile filtration unit (Sartorius SM 16510). The sterile solution collected from each plant was stored separately in the icebox at 5 °C in plastic jars.

The exudates from each replicate were tested individually for their hatch stimulus activity at 25 % dilution with 25 cysts in four replicates in glass vials. The number of emerged juveniles as well as the number of eggs and juveniles remaining in the cysts was determined after 2 weeks and the percent emergence calculated.

Penetration was determined by staining the nematodes in roots in an acid fuchsin — lactic acid solution in a microwave oven (Hooper, 1990). The roots were blended 2 × 20 s at 21 000 rpm in a Waring Blendor (Bender & Hobein) and the number of juveniles that had penetrated the root tissue determined using a stereo microscope (100 ×).

*Fungal egg colonization as affected by cyst age and time*

Results from our studies (Schuster & Sikora, 1992) demonstrated, that the level of infection of *G. pallida* eggs from the greenhouse stock culture was consistently below 2 %. The level of infection in controls with application of granules without fungus was equally low. Controls with fungus free granules were not used in the following experiments.

The number of newly formed *G. pallida* cysts and the level of infection with the same alginate treatment, application time and application rate from different own experiments were compared statistically using nonlinear regression. This procedure was used, because of the large degree of variation in level of egg infection existing between the experiments.

The Fs-alginate formulated granules were applied at 1 % w/w at planting to soil inoculated with 9 month-old cysts (1500 eggs and J2/100 g of soil). Eight 11 cm plastic pots were filled with 600 g of the treated soil and planted with one potato tuber. After eight weeks in the greenhouse, the females and cysts were extracted and then divided into three groups : 1) 9 month old cysts — those used initially as inoculum, 2) beige cysts, and 3) white females — the latter two groups newly formed during the course of the 8 weeks test period. The white females, beige cysts and 9 month old cysts from each pot were then examined separately for level of Fs egg infection as described before.

To study the ability of Fs in alginate granules to infect eggs of different ages, infection of eggs of 9 month old cysts and those in white females were compared. Hundred grams of soil per replicate were treated with 1 % Fs-alginate granules and inoculated with fifteen newly formed white females containing eggs or 9 month old cysts. The treatments were replicated four times and the level of infection determined after 2 weeks.

*Duration of infectivity*

Duration of activity of the sterile isolate (Fs) in field soil was determined by adding 1 % (w/w) alginate granules containing Fs per replicate to 100 g of field soil. Fifteen cyst/replicate were inoculated 0, 1, 2, 3, 4, 6 or 8 weeks after application. Each treatment was replicated four times and fungal egg infection was determined 2 weeks after cyst inoculation.

The duration of fungal infectivity was examined by two approaches : 1) 100 g of field soil containing 1 % Fs-alginate granules was inoculated with fifteen nine-month-old cysts and the cysts removed after 2 or 8 week intervals and the level of infection and number of eggs/cyst determined or 2) the cysts were extracted from the soil after 2 weeks exposure to the fungus, reinoculated into field soil without Fs-alginate inoculum and infection and number of eggs/cyst determined 8 weeks after the first exposure to the fungus. The treatments were replicated ten times.

*Fungal growth through field soil*

The ability of the fungus to overcome natural fungistasis, grow a specific distance from the inoculation point of the granule and still infect nematode eggs was measured using a template system designed by Sikora *et al.* (1990). Using the template, the cysts were placed at predeterminated distance of 0, 0.5 and 1 cm from the Fs-alginate granules. The level of infection was determined after 3, 7, 10 or 14 days as already described. Each distance was tested with 15 cysts and 20 granules in one Petri-dish and each distance replicated four times.

## Results

EFFECT OF FS-ALGINATE GRANULE FORMULATION ON ROOT WEIGHT, EMERGENCE AND PENETRATION OF POTATO

The root weight of potato was drastically reduced 76.4 % (ABNF 82.5 %; ABN 77.4 %; AF 69.9 %; A 75.6 %) over the untreated control 2 weeks after application following the application of alginate granules with or without Fs (Table 1). There were no significant differences between the individual alginate treatments. However, the negative effect on root weight was not evident after 8 weeks in those treatments containing additional nutrients (ABNF and ABN) when compared to the control. Conversely the root weight in the treatments without nutrient (AF and A) were still significantly lower after 8 weeks.

Similar results were obtained in hatching tests conducted with exudates collected 2 weeks after planting from the same experimental pots (Table 1). Emergence was reduced 89.2 % (ABNF 94 %; ABN 89.2 %; AF 86.5 %; A 87.1 %) in the alginate treatments *vs* the control. There were no differences in emergence between the alginate treatments.

**Table 1.** Effect of different nutrients and the sterile fungus (Fs) in alginate granules on root weight 2 and 8 weeks after application, on hatch and on penetration of *G. pallida*. Root weight (g).

| Control | 3.97 *a* | 6.90 *a* | 44.4 *a* | 1632 *a* |
|---|---|---|---|---|
| Treatments | 2 weeks | 8 weeks | hatch (%) | penetration |
| ABNF | 0.70 *b* | 6.54 *a* | 6.0 *b* | 181 *b* |
| ABN | 0.87 *b* | 8.12 *a* | 10.8 *b* | 140 *b* |
| AF | 0.95 *b* | 2.89 *b* | 13.1 *b* | 198 *b* |
| A | 0.64 *b* | 3.66 *b* | 12.9 *b* | 330 *b* |

Within a column, means followed by the same letter are not significantly ($p = 0.05$) different according to Duncan's Multiple Range Test.

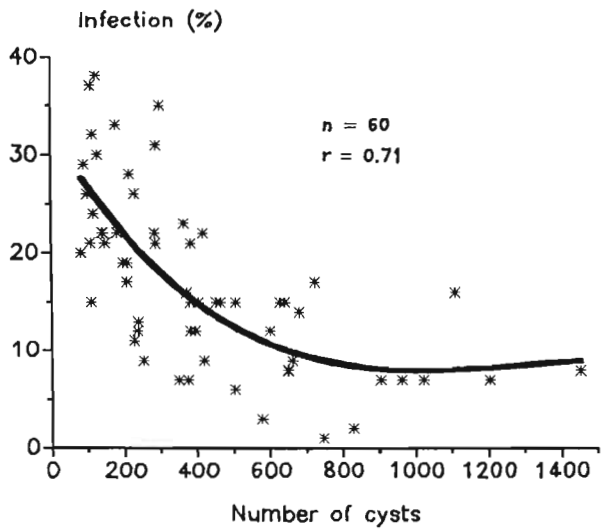


**Fig. 1.** Correlation between the number of newly formed *G. pallida* cysts and the degree of egg infection in different pot experiments with the sterile fungus (Fs) at the same amount of alginate granules applied ( r = 0.71; n = 60).

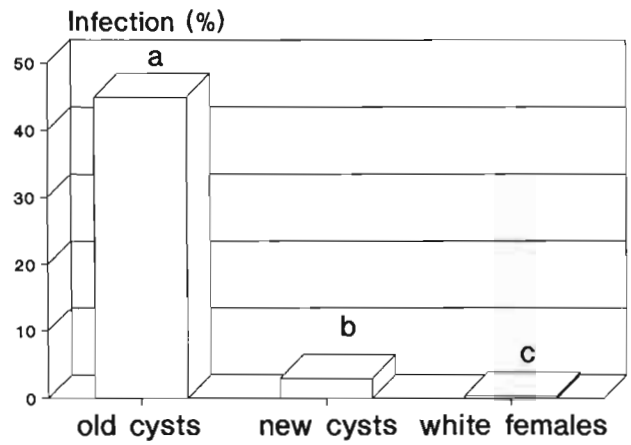


**Fig. 2.** Effect of the sterile fungus (Fs) applicated at planting in alginate granules on the degree of egg infection of inoculated 9 month old cysts, on the newly formed brown cysts and on white females of *G. pallida* 8 weeks after application. Means with the same letter are not significantly different (p = 0.05) according to Duncan's Multiple Range Test.

Comparable results were obtained in the penetration tests (Table 1). A distinct reduction of 82.2 % (ABNF 89.2 %; ABN 91.5 %; AF 85 %; A 62.9 %) was measured in the alginate granule treatments when compared to the control.

### Dynamics of colonization

The results from different own experiments with the same alginate treatments, application time and application rate were compared statistically using nonlinear regression. There was a clear negative correlation between the number of newly formed cysts and level of infection. Infection of more than 20 % was only detected when less than 500 cysts per replicate were present in the pots (Fig. 1).

When the Fs isolate in alginate granules was applied at planting, and the cysts in the tests were divided into three groups as to age, a distinct trend was detected. There was a definite difference between the infection levels with the Fs isolate in eggs in newly formed cysts and white females *vs* 9 month-old cysts (Fig. 2). Fungal infection, determined 8 weeks after application, showed that the newly formed beige colored cysts and white females had infection rates of 3 % and 0.38 % as compared to 45 % detected in the 9 month-old cysts initially used as inoculum.

The hypothesis generated in the previous test, that the fungus was unable to infect the eggs in females or newly formed cysts was not verified in the study with 9 month old cysts and newly formed white females with eggs. The results demonstrated that approximately the same percentage 28.8 and 29.5 % of eggs in the young and old cysts were infected, respectively (Fig. 3).

The incorporation of cysts into Fs-alginate treated soil at different time intervals after fungal application demonstrated clearly that the level of infection of the eggs was not due to difference in the age of eggs in the inoculum. The data showed that the Fs isolate is only able to infect eggs effectively for 3 weeks in field soil after inoculation in alginate at 1 % w/w. There is a definite decrease in the level of infection of eggs after 28 days in field soil (Fig. 4).

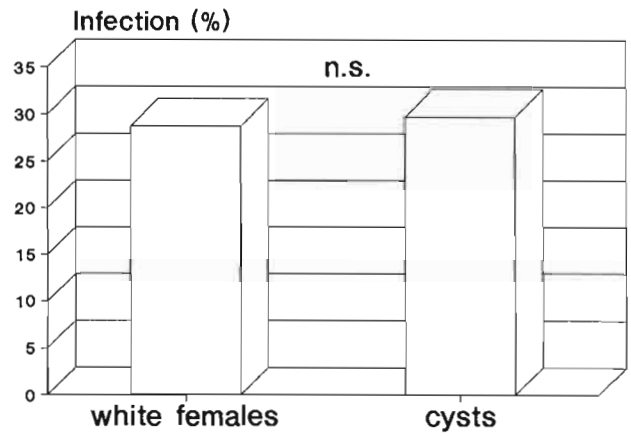


**Fig. 3.** Effect of the sterile fungus (Fs) in alginate granules on the egg infection of 9 month old cysts and white females of *G. pallida* 2 weeks after application (n.s. = not significant).

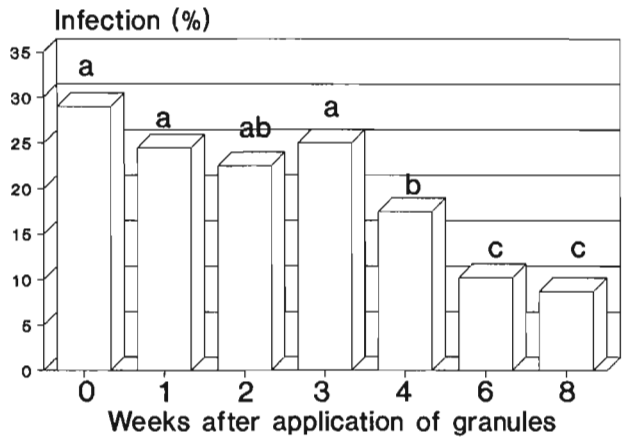


**Fig. 4.** Effect of time between application of the sterile fungus (Fs) in alginate granules and the inoculation of *G. pallida* cysts on the degree of egg infection. Means followed by the same letter are not significantly different (p = 0.05) according to Bonferoni - Holm Test.

The results from the experiments involving different durations of fungal exposure verified this dynamic process. In Figure 5, all treatments show equal levels of infection (29.5-30.5 %). Of importance is the treatment 2-8, because the cysts were separated from the fungal inoculum after 2 weeks of exposure. The number of eggs/cyst in the treatments examined after 8 weeks was approximately 30 % lower than the counts after 2 weeks. Hatching was not observed during the experiment.

The importance of a nutrient base for Fs survival and spread through the soil was demonstrated in the studies

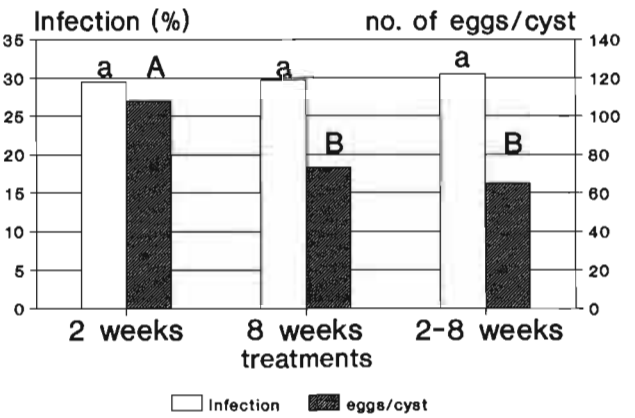


**Fig. 5.** Egg infection by the sterile fungus (Fs) and number of eggs/cyst of *G. pallida* dependent on the time of examination and the duration of fungal presence in soil. Means, separately for egg infection and number of eggs/cyst, followed by the same letter are not significantly (p = 0.05) different according to Duncan's Multiple Range Test.

where the distance between the cysts and the Fs-alginate granules were exactly controlled. Fs infection levels of up to 67.5 % were detected after 14 days when the cyst were in direct contact with the Fs-alginate granule (Table 2). The maximum level of infection at distance of 0.5 and 1.0 cm between the granule and the cyst of 30 % occurred after 7 days. Infection levels did not increase with time thereafter.

**Table 2.** Effect of time and distance of granule from cyst on infection of *G. pallida* eggs by the sterile fungus (Fs) applied in alginate granules. (Degree of infection (%).)

| | Time after application of alginate granules | | | |
|---|---|---|---|---|
| Distance (cm) | 3 days | 7 days | 10 days | 14 days |
| 0 | 30.3 *d* | 47.5 *b* | 60.0 *a* | 67.5 *a* |
| 0.5 | 18.5 *e* | 36.8 *cd* | 36.0 *cd* | 39.8 *c* |
| 1.0 | 18.5 *e* | 37.0 *cd* | 35.8 *cd* | 33.5 *cd* |

Means followed by the same letter are not significantly (p = 0.05) different according to Bonferoni - Holm Test.

## Discussion

The results showed a direct influence of alginate used in the formulation of egg pathogenic fungi on initial root growth patterns. Similar results were obtained by Lohmann (1989) with endoparasitic fungi in alginate granules for control of *G. pallida*. The reduction in root growth does not seem to be due to direct toxic effects on root tissue but to a retardation in germination or sprouting of the tuber. Root growth may be reduced by extensive fungal and bacterial growth around the granule, which could alter the $CO_2/O_2$ ratio leading to initial suppression of germination and root extension.

The negative effects of alginate on root growth is not detectable after 8 weeks. In this time interval the nutrients in the granule are metabolized by the activity of the fungal egg pathogen and associated soil microflora. This loss in nutrient availability from the alginate granule with time is also reflected in the loss of fungal infectivity of eggs after 4 weeks as well as in the visible reduction in the size of the granule after 8 weeks.

The combined reduction in root weight, root exudate levels and therefore hatch probably is responsible for the decrease in penetration in the alginate treatments. Rawsthorne and Brodie (1987) showed reduced hatching with smaller root systems. Furthermore Perry and Clarke (1981) demonstrated that penetration is directly affected by factors altering hatching rate. The reduction in newly formed cysts detected by Schuster and Sikora (1992) when fungal egg pathogens were applied to field

soil in alginate were probably also due to the negative effects of alginate on root growth.

Our hypothesis that alginate based fungal formulations applied at the time of planting could be effective in biological control of the eggs in newly formed cysts proved to be invalid. Neither regression of infection rate to number of cysts nor additional experimentation showed significant levels of infection of eggs in newly formed cysts. The lack of significant fungal growth in the soil after 4 weeks in the control tests clarifies why control after completion of the life cycle is impossible. The fungus has the ability to grow quickly out of the alginate granule into the soil when supplied with nutrients, but is then suppressed with time by other competitors. The sensitivity of the isolate to fungistasis may lie in its sterile non-sporulating characteristics, in contrast for example to *Verticillium chlamydosporium* that produces chlamydospores.

The results of test comparing infection between 2 and 8 weeks after fungal application in alginate, demonstrated, that an in-cyst infection dynamic leading to increasing rates of egg infection does not take place. The level of infection within the cyst in this time interval is not dependent on the presence of the fungus in the soil, as demonstrated by the fact that the same levels of infection were detected when cysts were constantly exposed to the fungus for 8 weeks or were separated from the fungal inoculum after 2 weeks exposure.

However, there was a significant reduction in the number of eggs and juvenile per cyst after 8 weeks when compared to 2 weeks exposure, because the eggs infected after 2 weeks are degraded and no longer detectable. Knuth (1986) showed that *V. chlamydosporium* completely colonized the eggs of *Heterodera avenae* after 30 days at 22 °C. The theoretical in-cyst dynamics of fungal development in *H. schachtii* eggs was proposed by Nicolay (1989) and is considered a slow process. The reduction in number of eggs per cyst in our experiment can not be due to hatch, because *G. pallida* hatches only in the presence of host root exsudates (Perry, 1986).

The use of the templet for precise measurement of fungal growth through soil and determination on of infection as influenced by time of exposure and distance between fungal inoculum and host, demonstrated that the maximum infection levels were reached over long distances after 7 days, regardless of initial inoculum density. The results also showed that infection levels increased drastically when the cyst were placed directly adjacent to the fungal inoculated alginate granule. The fact that infection levels did not increase progressively indicates an other mechanism of infection and not true parasitism. In addition, infection levels increased progressively with increasing inoculum levels. The results demonstrate the presence of fungal based toxic metabolites that initially cause egg mortality, with the fungus most probably entering the egg after death. Nicolay and Sikora (1989) suggested that many fungi detected in eggs are growing saprophytically on dead tissue which may have been initially killed by perthotrophic behaviour. Sikora *et al.* (1990) discussed the different definitions of egg colonization and discussed the perthophytic mode of infection associated with facultative pathogens.

The infection rates in our tests were consistently near 30 %. The only exception beeing those treatments where the cysts were placed directly adjacent to the alginate granule. This indicates that only a certain portion of the eggs in the cyst are highly suspectible to infection at any one time, and probably indicates the colonization of non-viable eggs present in the cyst.

Our results demonstrate some of the advantages and limitations of using fungi for the biological control of *G. pallida* on potato. The data confirmed earlier findings, that infection of *G. pallida* eggs by the fungus was based on a perthophytic mode-of-action. Alginate as a test formulation, although causing some initial germination and root growth inhibition, proved to be an elegant technique for studying biological control and fungal behaviour toward cyst nematodes in field soil. Furthermore, the results showed that application of a perthophytic pathogen in alginate 8 weeks prior to planting can increase biological control by reducing the number of viable eggs in dormant cysts, thereby reducing nematode early root infection. The presence of toxic metabolites produced by fungal pathogens is often overlooked in standard bioassay tests for biological control agents. Bioassay systems used for detection of fungal egg pathogens or parasites should be examined for their suitability to detect organisms with predetermined mode-of-action. The mechanism by which parasitism or pathogenicity occurs beeing important for optimizing biocontrol.

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