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# Influence of different formulations of fungal egg pathogens in alginate granules on biological control of *Globodera pallida*

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**Summary** — Application of *Acremonium sordidulum* and a sterile fungus (Fs) in alginate granules to unsterile field soil significantly decreased the number of newly formed cysts of *Globodera pallida*. The proportion of eggs colonized by both fungi was greater in the fallow soil treatment than in the treatment with a host plant. Application of Fs in alginate granules at densities of 0.5 and 1 % (w/w) caused significant reductions in the number of newly formed cysts and egg colonization increased significantly at all concentrations except 0.1 % (w/w). There was no relationship between the number of newly formed cysts and increased Fs inoculum density/alginate granule. The same levels of egg colonization and reduction in the number of newly formed cysts was obtained with granules of different sizes. With one exception, all dates of application caused significant reductions in cyst number and egg colonization increased in almost all cases. There was a reduction in the number of newly formed cysts following application of alginate granules without fungus.

**Résumé** — ***Influence sur le contrôle biologique de* Globodera pallida *de différentes formulations de granulés d'alginate contenant des champignons attaquant les œufs*** — L'application d'*Acremonium sordidulum* et d'un champignon stérile (Fs) contenus dans des granulés d'alginate à un sol cultivé non stérilisé diminue significativement le nombre des kystes néo-formés de *Globodera pallida*. La proportion d'œufs colonisés par les deux champignons est plus élevée pour un sol sous jachère que pour un sol avec une plante hôte. L'application de Fs dans les granulés d'alginate à la densité de 0,5 à 1 % (poids pour poids) produit une diminution significative du nombre de kystes néo-formés et la colonisation des œufs est significativement accrue pour toutes les concentrations à l'exception de 0,1 % (poids pour poids). Il n'y a aucune relation entre le nombre de kystes néo-formés et l'accroissement de l'inoculum de Fs dans les granulés d'alginate. Les mêmes niveaux de colonisation des œufs et de réduction des kystes néo-formés sont obtenus avec des granules de tailles différentes. Sauf une exception, la date d'application est indifférente et la réduction du nombre de kystes néo-formés ainsi que l'augmentation de la colonisation des œufs se produisent dans presque tous les cas. Une réduction du nombre des kystes néo-formés est également observée après application de granulés d'alginate dépourvus de champignons.

**Key-words :** *Globodera*, biological control, nematode pathogenic fungi, alginate, *Acremonium*.

The potato cyst nematode *Globodera pallida* (Stone) an important quarantine pest in Germany, can cause severe losses and in some countries the yield is smaller than the amount of potatoes planted (Mai, 1977). Methods presently recommended for integrated control include : longer rotations with non-hosts, resistant cultivars and nematicides. Each technique has certain disadvantages : 1) extending the interval between potato crops is often uneconomical, 2) nematicide use is prohibited in growing areas in water conservation zones, 3) yield of many resistant cultivars is not comparable to that of susceptible cultivars, and 4) only one cultivar is resistant to race 3 (Heinicke & Maykuhs, 1982; Behringer, 1988). The development of biological control could be an acceptable component of an integrated control program, being theoretically both environmentally safe as well as effective toward all races.

Although only a few studies have been conducted on fungal colonization of *Globodera* eggs (Dackman, 1990), a number of fungi have been shown to effectively infect their eggs. Roessner (1987) isolated *Cladosporium herbarum, Preussia* sp. and two isolates of *Fusarium oxysporum* from *Globodera rostochiensis* eggs in Germany. Hiemer and Sikora (1988*b*) isolated large numbers of fungi from *G. pallida* eggs. Initial attempts by Hiemer and Sikora (1988*a*) to incorporate these isolates into field soil as mycelial/spore suspensions produced in liquid shake cultures or on colonized straw for biological control of *G. pallida* were consistently unsuccessful. Conversely, 27 % of the *G. pallida* eggs examined were colonized when a bran : sand formulation containing a sterile fungus was applied to unsterile field soil (Hiemer & Sikora, 1988*a*). None of the formulations produced economically acceptable levels of control when compared to chemicals or resistant cultivars. They concluded that effective biological control required the development of an acceptable formulation for application to soil that ensures fungal establishment and high levels of egg infection and therefore of control.

The utilization of alginate, a product of the food

processing industry, was first described by Walker and Connick (1983) as a formulation for myco-herbicides and was subsequently used by other researchers (Lewis & Papavizas, 1985; Fravel *et al.*, 1985; Mauperin *et al.*, 1987; Cabanillas *et al.*, 1989). In our research program we attempted to use alginate to formulate fungal isolates with a known ability to infect *G. pallida* eggs. The goals of our investigations were to : 1) study the effect of fungal antagonists applied in alginate granules on egg infection of dormant cysts under fallow conditions or on penetration and cyst production as well as egg production in the presence of a host plant, 2) determine optimum application rates, using both amount of granules and by increasing fungal density in the granules, 3) optimize control by reducing granule size to increase distribution in soil, 4) increase control by delaying the time of application to coincide with initiation of egg-laying, and 5) study the influence of different nutrients incorporated into alginate granules on level of biological control.

## Material and methods

### General methods

*Preparation of nematode infested soil*

The substrate used was a 1:1 (v/v) mixture of a cyst nematode free, nonsterile field soil and sand. Small amounts of nematode infested soil/sand substrate from greenhouse culture pots were added to the field soil/sand mixture to give a final infestation of 1500 eggs and juveniles (in cysts)/100 g soil. The nematodes in their original culture pots were stored at 10 °C for 8 months prior to use as inoculum, to ensure adequate emergence and penetration. The *G. pallida* population used was predominantly pathotype-1.

In the experiments glasshouse plastic pots 11 cm in diameter with a capacity of 600 g soil were used. Certified seed tubers cv. Hansa, 3.5 cm in diameter were used in all tests.

*Source and production of fungi*

*Acremonium sordidulum* (As) and a nonsporulating isolate resembling *Fusarium* (Fs), both isolated from *G. pallida* eggs, were used in the experiments.The fungi were selected because of their ability to infect > 90 % of eggs in agar bioassay tests (Hiemer & Sikora, 1988*b*; Sikora, *et al.*, 1990). The fungi were cultivated on 1 % potato dextrose agar (PDA) in Petri dishes at 25 °C for 5 days in incubators. Inoculum was produced by carefully scrapping the fungal mycelium from the surface of one 5 day old Petri dish and adding this as inoculum to 500 ml of a modified glycerine-peptone solution. The solution was incubated in 1000 ml glass flasks at 21 °C for 7 days on a rotary shaker. Approximately 40 g of fungal fresh weight was produced in each flask.

*Production of alginate granules*

The alginate granules were produced by modifying the technique of Walker and Connick (1983). A nutrient solution (described below) with or without fungus was homogenized with a Ultra-Turrax (IKA-Werk) for 30 s at 20 000 rpm. The resulting solution was combined with 2 % (w/w) Na-Alginate pract. (Fluka) and then thoroughly homogenized with a Ultra-Turrax for 30 s at 20 000 rpm. The final solution was passed through a pipet with 2 mm diameter end opening with pressure from an air pump and the droplets collected in a basin containing a 0.25 m $CaCl_2$ solution (Fravel *et al.*, 1985; Lewis & Papavizas, 1985). The alginate granules were left in the $CaCl_2$-solution for 45 min to allow for complete polymerization (Maupertin *et al.*, 1987) and then dried in trays at 20 °C in the laboratory for 2-3 days. The dried granules were stored in sealed jars in the dark at 4 °C and used within 7 days.

With the exception of the experiment with different nutrient additives, 920 ml a glycerine-pepton-solution in addition to 2 % (w/w) of milled wheat bran was used as the nutrient solution for the granules. Nutrient solutions for other formulations are listed in Table 1. All granules with fungal inoculum contained a total of 80 g fresh weight (1.7 g dry weight) of 7 day old fungal cultures produced on a rotary shaker (ABNF-type, see Table 1.).

**Table 1.** Nutrient combinations incorporated into alginate granules either with or without the fungus.

| *Code Name* | *Nutrient combinations* | *Fungal Inoculum* |
|---|---|---|
| ABN | Glycerine-peptone solution + wheat bran | — |
| ABNF | Glycerine-peptone solution + wheat bran | + |
| AN | Glycerine-peptone solution | — |
| ANF | Glycerine-peptone solution | + |
| AB | Distilled water + wheat bran | — |
| ABF | Distilled water + wheat bran | + |
| A | Distilled water | — |
| AF | Distilled water | + |

Both fungi As or Fs were incorporated singly as mycelium into the granules. The nutrients and additives used in the granules are listed in Table 1 with their acronyms. The average weight of each granule was 3 mg. Approximately 330 granules/100 g soil were applied for treatments of 1 % (w/w). Each granule contained an average 0.005 g fresh weight (0.0001 g dry weight) of fungus. At an application density of 1 % (w/w), 1.7 g/100 g soil of fungal fresh weight (0.035 g dry weight) was applied.

The amount of fungus growing in the soil could not be determined at application time because the fungus remains dormant in the granules until an unknown amount of water uptake takes place. After granule rehydration, fungal growth out of the granules as

mycelium occurs very quickly and can reach cysts at a distance of 2 cm and infect the eggs (Sikora *et al.*, 1990).

*Evaluation*

Initial and final cyst densities were determined by the wet-sieve decantation technique. Organic material was separated from the resulting cyst suspension using a 1285 g/ml $MgSO_4$ solution. The number of cysts were counted and egg and juvenile numbers determined by separating the eggs from cysts by crushing with a hand held tissue homogenizer. The eggs were then collected on a 20 µm sieve and concentrated in centrifuge tubes by centrifugation at 2500 rpm (Hettich universal II) for 8 min. The supernatent was decanted until 4.5 ml remained and 0.5 ml of a 1 % chloramphenicol solution added to each tube. A 0.5 ml aliquot sample was then spread onto a 1.5 % water agar Petri dish. The agar dishes were incubated at 21 °C for 24 h. The level of infection was determined by examination of 100 eggs for mycelial growth under a microscope at 100 ×.

The experiments were conducted in the glasshouse or in environmental chambers at a mean temperature of 21 °C with 16 h of additional artificial light. The experiments were terminated after 8 weeks or after completion of the nematodes life-cycle.

EXPERIMENTAL DESIGN

*Incorporation in alginate under fallow or with host plant*

*A. sordidulum* (As) or the sterile fungus (Fs) incorporated into alginate granules were used in this experiment. The granules were applied at a concentration of 1 % (w/w) at planting by first mixing the total amount of granules for each treatment before potting with the total amount of soil needed for the replicates of each treatment. In this experiment, the ABNF granules (Table 1) were used. Each treatment was replicated six times and the experiment was terminated after 8 weeks.

*Time of application*

ABNF granules (Table 1) containing As or Fs were used in this experiment, and were applied to soil at planting at a concentration of 1 % (w/w), as already described. Applications of 2, 4 or 6 weeks after planting were made by carefully removing the potato plants at the desired treatment time from the pots, mixing the soil left in the pot with the appropriate amount of granules (1 % [w/w]) and replanting the potato plants into the newly treated soil. The pots were left in the growth chambers for 8 weeks after which the experiment was terminated.

*Alginate granule application rate*

Application rates of ABNF granules containing Fs (Table 1) of 0, 0.1, 0.3, 0.5, 1 % (w/w) in soil were used and applied at planting by mixing them with the total amount of soil as already described. The experiment was conducted with eight replicates/treatment and terminated after 8 weeks.

*Fungal concentration in granules*

The required 40, 80, 160 or 320 g mycelial fresh weight (0.8, 1.6, 3.2, 6.4 g dry weight) of the Fs fungus was produced in glycerine-peptone broth. The fungal mycelium was separated from the broth on a 100 µm sieve, washed and weighed and the desired amount of fresh mycelium added to freshly prepared nutrient solution to give a final volume of 1000 ml. The resulting solution was incorporated into the alginate granules as already described. The granules used in this experiment were the ABNF type and were applied at a concentration of 1 % (w/w) at planting. The treatments were replicated eight times and evaluated 8 weeks after planting.

*Powder alginate formulations*

Granules of the ABNF type (Table 1) containing Fs, were used which were either left unground or were ground in a electric mill to a particle size of < 1 mm prior to treatment. The two formulations were applied at planting or under fallow conditions at a rate of 1 % (w/w) with eight replicates/treatment. In both cases, the experiments were terminated after 8 weeks.

*Nutrient additives in alginate*

The sterile fungus (Fs) was incorporated into alginate granules with different nutrient additives. The nutrient combinations incorporated into the alginate granules are listed in Table 1. The granules were applied at a concentration of 1 % (w/w) at planting with eight replicates/treatment and the test terminated after 8 weeks.

The nutrient free inoculum in the treatments AF and ABF was prepared by separating the culture solution through a 100 µm sieve to remove the nutrient solution and adding an equal amount of distilled water in its place.

## Results

INCORPORATION IN ALGINATE UNDER FALLOW OR WITH HOST PLANT

When alginate granules were applied at planting the number of newly formed cysts decreased significantly in the formulations with *A. sordidulum* (As) and the sterile fungus (Fs) 63.5 % and 69.7 % over the control (Table 2). The proportion of eggs infected by both fungi was greater in the fallow soil treatment than in the presence of potato. Significantly higher levels of infection were only measured in the As treatment (Table 2). Although the rate of biological control with Fs was higher under fallow and in the presence of potato than with As, the difference between the two formulations was not statistically different. Important was that neither fungus, regardless of treatment type, affected fecundity measured as the number of eggs and juveniles/cyst.

TIME OF APPLICATION

With one exception, all times of granule application

**Table 2.** Effect of *A. sordidulum* (As) and a sterile fungus (Fs) in alginate granules on fungal egg infection and number of newly formed *G. pallida* cysts.

| | Degree of infection (%) | | Number of cysts |
|---|---|---|---|
| treatments | fallow | potato | potato |
| control | 7 c | 3 c | 799 a |
| *A. sordidulum* (As) | 26 a | 15 b | 292 b |
| sterile fungus (Fs) | 28 a | 21 ab | 242 b |

Treatments followed by the same letter are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

caused significant reductions in the number of cysts over the untreated control (Fig. 1).

Application of granules containing Fs caused significant control of 47, 53.4 and 58.5 % at planting as well as at 2 and 4 weeks after planting. Large reductions in the number of cysts of 70.2 and 46.1 % were also obtained with granules containing As 2 and 4 weeks after planting, respectively. The treatments did not affect the number of newly formed cysts if applied 6 weeks after planting.

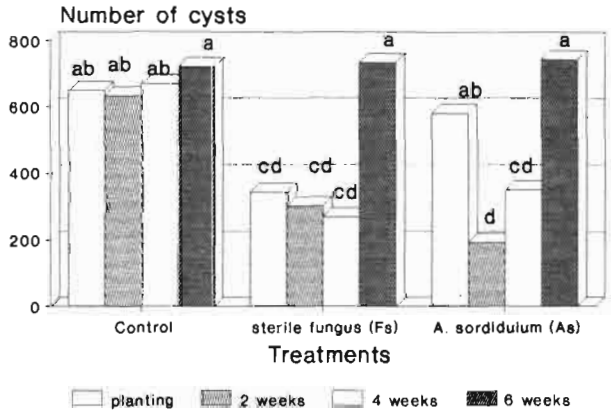


**Fig. 1.** Effect of time of application of alginate granules containing *A. sordidulum* (As) and the sterile fungus (Fs) on the number of newly formed *G. pallida* cysts. Columns with the same letters are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

Fungal infection of the eggs increased in almost all cases, independent of time of application, when compared to the untreated control (Fig. 2). Furthermore, degree of infection decreased as time between planting and application increased. The only exception was a 9 % infection rate when As was applied at planting. The highest levels of control were reached in treatments with the Fs isolate, whereby application at planting and 6 weeks later were significantly different from treatments with As. Fecundity was not significantly affected by fungus isolate or time of application.

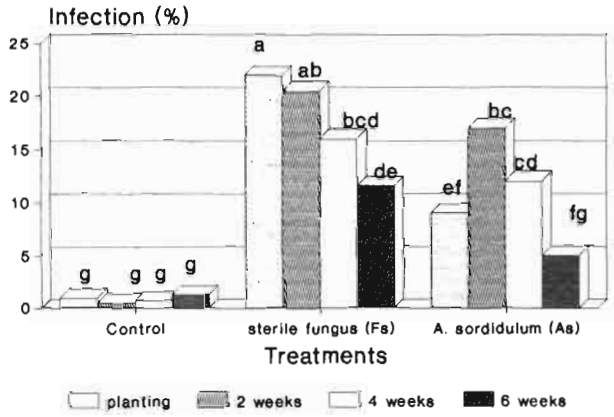


**Fig. 2.** Effect of time of application of alginate granules containing *A. sordidulum* (As) and the sterile fungus (Fs) on fungal infection of *G. pallida* eggs. Columns with the same letters are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

Because the results of the first two experiments demonstrated that the level of biological control of *G. pallida* was consistently greater in the Fs treatments than in the As treatments, the remaining experiments were conducted only with Fs.

### Alginate granule application rate

The application of alginate granules with Fs at planting at densities of 0.5 and 1 % caused significant reductions in the number of newly formed cysts when compared to the control (Fig. 3). The degree of infection of *G. pallida* eggs by Fs increased significantly over the control at all concentrations except 0.1 % (Fig. 3).

### Fungal concentration in alginate granules

There was no significant relationship between the number of newly formed cysts and Fs inoculum density/alginate granule (Fig. 4). Important was the lack of a statistically significant difference between the alginate treatment in the control without fungus and that with fungus. The level of infection of *G. pallida* eggs increased significantly with increasing levels of fungal inoculum. The number of eggs/cyst was not affected.

### Powdered alginate formulation

The Fs isolate, in both granulate (> 3 mm) and powder form (< 1 mm), caused significant increases in egg infection in old cysts under fallow (Fig. 5). In the presence of the host, they caused significant differences in the rate of infection only when compared to the untreated control (Fig. 6). In the latter case 75 %

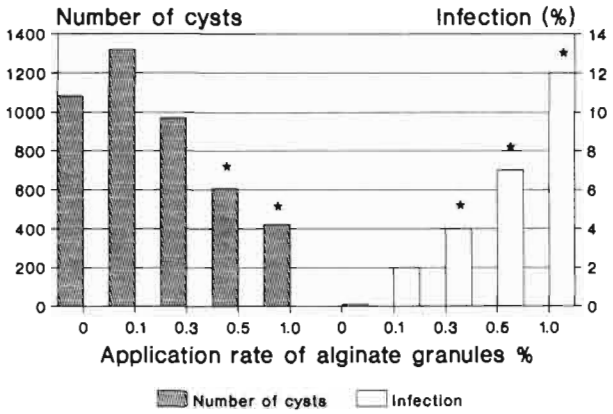


**Fig. 3.** Effect of application rate of alginate granules containing the sterile fungus (Fs) on the number of newly formed *G. pallida* cysts and fungal egg infection. Significant differences from the control according to LSD ($P < 0.05$) marked *.

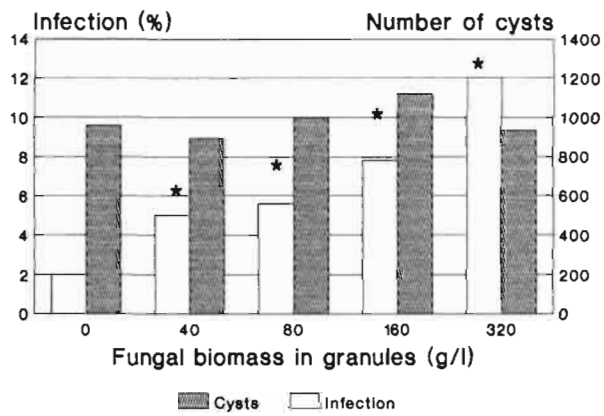


**Fig. 4.** Effect of concentration of the sterile fungus (Fs) in granules on the number of newly formed *G. pallida* cysts and fungal egg infection. Significant differences from the control according to LSD ($P < 0.05$) marked *.

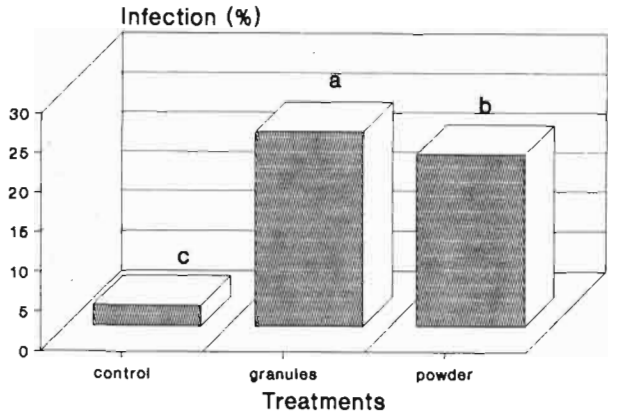


**Fig. 5.** Effect of alginate granules or powder, containing the sterile fungus (Fs), on the fungal infection of *G. pallida* eggs under fallow. Columns with the same letters are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

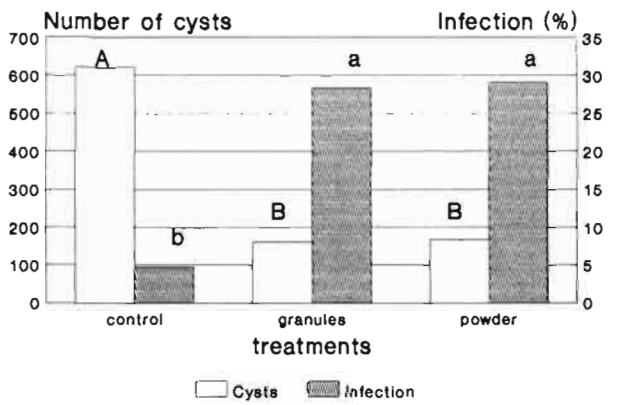


**Fig. 6.** Effect of alginate granules and powder containing the sterile fungus (Fs) on the number of newly formed *G. pallida* cysts and fungal egg infection in the presence of a host. Columns with the same letters are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

reduction in the number of newly formed cysts was detected with both formulations (Fig. 4). Neither treatment affected *G. pallida* fecundity.

### Nutrients additives in alginate

There was a consistent reduction in the number of newly formed cysts following application of alginate granules containing nutrient additives with or without fungus when compared to the control (Table 3). Differences in the number of cysts formed could not be detected within alginate treatments with or without the fungus or between treatments with or without different nutrient additives.

The smallest number of cysts was detected in the A and AF treatments and was most probably caused by strongly reduced root growth (Table 3). Conversely, there were no significant differences between root weights in the other treatments or in the control.

As expected, fungal infection of *G. pallida* eggs was significantly greater in the treatments with fungi than in those without fungi or in the control (Table 3). The ABNF treatment, which contained the highest level of nutrients and was the standard treatment used in our tests, induced the highest rate of fungal egg infection in the treatments with fungi. Conversely, the level of natural infection in the controls without fungus only

**Table 3.** Effect of different nutrients and the sterile fungus (Fs) in alginate granules on root weight, number of cysts and fungal infection of *G. pallida* eggs 8 weeks after application.

| Treatment | | Root weight (g) | Cyst. no. | Colonization (%) |
|---|---|---|---|---|
| | 0 | 6.90 bc | 1569 a | 1.8 c |
| without fungus | A | 3.66 de | 309 b | 3.0 c |
| | AB | 6.96 bc | 692 b | 2.8 c |
| | AN | 9.23 a | 661 b | 3.1 c |
| | ABN | 8.12 ab | 665 b | 3.0 c |
| with fungus | AF | 2.89 e | 379 b | 9.0 b |
| | ABF | 4.94 cd | 744 b | 9.5 b |
| | ANF | 5.90 c | 434 b | 10.4 b |
| | ABNF | 6.54 bc | 643 b | 14.8 a |

Treatments followed by the same letter are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

approached 3 % and 1.8 % in the alginate and untreated control respectively. Nematode fecundity as observed in all previous experiments, was not affected.

## Discussion

The application of alginate granules containing the fungus *Acremonium sordidulum* (As) or the sterile fungus (Fs) led to significant reductions in the number of newly formed cysts and increased rates of fungal egg infection. Cabanillas *et al.* (1989) obtained similar results with *Paecilomyces lilacinus* applied in alginate granules. They stated that the highest level of egg colonization was reached with fungal application in alginate granules.

The reductions in the number of newly formed cysts of over 60 % over the untreated control in our study, can not be explained by fungal egg infection of 15-20 % obtained in our initial experiments. This is particularly true, because the rate of infection was even lower in the presence of a host plant. The results indicate the existence of other mechanisms-of-action in the fungus-alginate formulation that affect nematode development.

The results of the experiment on time of application clearly show, that application 6 weeks after planting does not have any effect on number of newly formed cysts. Six weeks after planting is the increament in time when white females appear at the root surface. This indicates, that the alginate treatments do not have a direct effect on the females or the fecundity of the females. In addition, the reduction in newly formed cysts in the other treatments can not be explained by the treatment effects on female development inside the root tissue and the low level of egg infection. The results indicate an additional mechanism of control which lead to a reduction in newly formed cysts. The As treatment demonstrate that the degree of eggs infected is related to cyst number and shows an inability to reduce the number of cysts formed by infecting the eggs.

Although the degree of egg infection increased two-fold when Fs granules were applied at planting opposed to 6 weeks after planting, in both cases white females occur on the root surface simultaneously. This indicates that the degree of infection detected following application at planting was caused by a high percentage of the infection of eggs in the initial inoculated cysts. Similar conclusions can be drawn for application 2 and 4 weeks after planting.

Increasing both granule application rate as well as fungal concentration in the granule demonstrated clearly that fungal density in the soil alone was responsible for the higher rates of egg infection. Similar results were obtained by Kerry *et al.* (1986) with *Verticillium chlamydosporium* and *Heterodera avenae.*

The fact that incorporation of increasing amounts of the fungus in the granules did not influence the number of newly formed cysts, demonstrated that the alginate used in formulation had adverse side-effects on nematode development. Whether this effect was : 1) indirectly caused by negative effects on the host plant 2) produced through stimulation of other soil microorganisms or 3) by direct action of specific components in the alginate on the nematode was not clear. Walker and Connick (1983) mentioned that alginate is used in food processing, because of the non-toxic characteristic of the substrate. Lohmann (1989) however, showed that alginate can have negative effects on early root growth and can cause significant increases in soil microbial activity.

The stability of the fungal-alginate formulation toward mechanical stress was shown in the tests with powdered granules. The same levels of control through egg infection as well as through a reduction in the number of newly formed cysts was obtained with both formulations at a concentrations of 1 % (w/w). The application rate used in the experiments is too high for a broadcast field application. However reduced granule size, increased fungal inoculum in the granules and in furrow or individual plant application may lead to economical levels of biological control.

Although the reduction in newly formed cysts was often shown to be due to indirect effects of alginate treatment on root growth, the presence of alginate in other treatments did not alter root growth. Reduced root weight in the alginate treatment without nutrients, demonstrated that reduced root growth is limited to the first few weeks after germination. In the treatments with nutrients, the roots recovered with time. The highest level of fungal infection of eggs was achieved in treatments with the addition of nutrients to the granules. Papavizas and Lewis (1981) mentioned that high nutrient supply is very important in overcoming soil fungistasis. In our studies, the nutrients in the alginate granules also may have suppressed fungistasis, thereby increasing efficacy through improved soil colonization.

We believe the alginate formulation can be used, as a model to determine biological control efficacy of fungi in field soil and to study the complex interrelationships between fungus, nematode and soil microflora in the rhizosphere.

Effective biological control may be achieved by developing application forms consisting of combinations of organisms in alginate granules or other carriers, each having a different mode-of-action. For example rhizobacteria (Sikora, 1988), fungal egg pathogens or parasites (Sikora *et al.*, 1990) and endoparasitic fungi (Lohmann & Sikora, 1988) that can utilize specific nutrient bases for establishment and growth could have potential in nematode biological control. Increasing the concentration of inoculum in the granules, selection of proper nutrient balance in favor of the fungus and reduction in granule size and a more targeted application to the plant could lead to successful biocontrol on high value cash crops.

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