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# Isolation of Fungi from *Heterodera glycines* and in vitro Bioassays for Their Antagonism to Eggs[1]

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*Abstract:* Twenty fungi were assayed in vitro for antagonism to eggs of *Heterodera glycines*. Eight of the fungi were isolated from cysts or eggs of *H. glycines* during the current study, one was isolated from *Panagrellus redivivus*, and eleven were obtained from other researchers or collections. The bioassays were conducted on eggs from nematodes that had been grown monoxenically on excised root tips. *Phoma chrysanthemicola*, one strain of *Verticillium chlamydosporium*, and one strain of *V. lecanii* caused a decrease ($P < 0.01$, $P < 0.05$, $P < 0.05$, respectively) in the number of viable eggs, although no hyphae were observed colonizing live eggs. *Trichoderma polysporum* infected live eggs but enhanced ($P < 0.05$) egg survival. *Acremonium bacillisporum*, *Chaetomium* sp., *Drechmeria coniospora* (two strains), *Epicoccum* sp., *Exophiala jeanselmei*, *Fusarium* sp., *Neocosmospora vasinfecta*, *Scytalidium fulvum*, *Trichoderma harzianum* (two strains), *V. chlamydosporium* (one strain), *V. lecanii* (three strains), and an unidentified fungus did not measurably affect egg viability, even though hyphae of five of these fungi were seen in live eggs. The bioassay provides a useful step in the selection of a biological control agent for this major nematode pest.

*Key words:* biological control, fungus–nematode interaction, *Heterodera glycines*, antagonist bioassay, soybean cyst nematode, *Acremonium bacillisporum*, *Chaetomium* sp., *Drechmeria coniospora*, *Epicoccum* sp., *Exophiala jeanselmei*, *Fusarium* sp., *Neocosmospora vasinfecta*, *Phoma chrysanthemicola*, *Scytalidium fulvum*, *Trichoderma harzianum*, *Trichoderma polysporum*, *Verticillium chlamydosporium*, *Verticillium lecanii*.

*Heterodera glycines* Ichinohe, the soybean cyst nematode (SCN), occurs in many soybean-producing countries (14). Most of the nematicides applied to control this pest are no longer registered in the United States (14). Consequently, successful biological control agents would be very useful for its management. No commercially produced biocontrol agent has wide application for such a program. Commercial formulations with potential to control SCN did not perform well in greenhouse and field tests (13). Despite such difficulties, positive results obtained with biocontrol fungi on nematodes in a number of other studies (11) indicate that continued isolation and testing of fungi should lead to a fungal agent that will aid in nematode control.

As part of the search for biocontrol agents, researchers have isolated fungi associated with *H. glycines* in the field (1,16). Association with SCN cysts, however, does not indicate that a fungus is pathogenic to the nematode, nor does lack of a known association mean that a fungus cannot affect the nematode (16). Fungi that are not known to parasitize SCN but that are antagonistic to other organisms may prove to be useful biocontrol agents for *H. glycines*. To find a successful biocontrol fungus, therefore, many species and strains of fungi should be tested for effectiveness against SCN. This challenge has resulted in the development of in vitro bioassays for antagonism to nematodes. Pathogenicity in vitro does not insure that a fungus will be an effective biocontrol agent, but screening fungi in the greenhouse and field consumes much time and space. As a consequence, prior selection of fungi for these studies is valuable. Furthermore, laboratory studies must be conducted even on known pathogens to determine mechanisms of pathogenicity. The objectives of this study were to develop an in vitro bioas-

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say for identifying fungi antagonistic to eggs of *H. glycines*, to determine whether selected fungi exhibited such antagonism, and to investigate whether deleterious effects were primarily produced by egg parasitism or by a noninfective mechanism.

## Materials and Methods

*Fungal isolation:* Fungi were isolated from cysts and eggs of *H. glycines* and from contaminated cultures of the nematode *Panagrellus redivivus* (L.) Goodey. Fungi were isolated from cysts and eggs by two procedures. The first method focused on fungi from field-grown cysts and eggs. Maryland field soil infested with race 3 of *H. glycines* was supplied by Dr. L. R. Krusberg of the University of Maryland. Soil was kept at 4 C until used. Cysts were extracted from soil samples by suspending the soil in water and washing the samples through nested 1,000-μm-pore and 250-μm-pore sieves. Cysts were surface-sterilized by a technique similar to that of Morgan-Jones et al. (12), except that cysts were washed twice in sterile distilled water after treatment with streptomycin. Cysts were placed in petri dishes and squeezed with forceps to force the eggs onto the agar. Cornmeal agar (CMA), 1.5% water agar (WA), potato dextrose agar (PDA), V-8 juice agar (V-8), peptone yeast extract glucose agar (PYG) (17), and chitin agar were used. Chitin agar was prepared by a modification of the method of Godoy et al. (4); the chitin-acid solution was not passed through dacron gauze. Water agar, PDA, and V-8 were prepared both with and without 0.025 g streptomycin sulfate (strep)/500 ml medium. Petri dishes containing eggs and cysts were placed in growth chambers at 15, 20, or 25 C to select for fungi with different optimal temperature requirements.

The second procedure was for the isolation of fungi from nematodes on greenhouse plants. These fungi were obtained by two methods. Cysts of *H. glycines* race 3 were collected from excised soybean (*Glycine max* (L.) Merr. cv. Kent) root tips grown on Gamborg's B-5 medium (3,5,9), placed between pieces of nylon mesh held together with metal frames, and buried in the soil for 1 week near roots of greenhouse-grown soybean plants. Fungi were also isolated from cysts and eggs produced on soybeans in pots. Cysts and eggs from both sources were either surface sterilized or left untreated and then placed on PDA + strep, WA + strep, V-8 + strep, or CMA and incubated at 25 C. Fungi from both the field and greenhouse were transferred after 2–13 days of incubation of the cysts and eggs. The fungi selected for bioassays were identified with the exception of one fungus that did not produce spores.

*Bioassay for antagonism to nematodes:* Assays were conducted on nine fungi isolated from nematode sources during the course of this study and on 11 fungi obtained from other researchers or fungus collections (Table 1). All fungi were maintained on PDA without streptomycin sulfate.

Eggs from mature yellow females and from brown cysts were studied separately. The mature yellow females were obtained from root explant cultures about 6 weeks old, and the brown cysts were obtained from root explant cultures that were at least 2.5 months old. Females and cysts were laboratory propagated and had external egg masses and internal eggs. A total of 48 yellow females and 48 brown cysts were used for each bioassay experiment. The 96 mature females and cysts were placed on 1.5% WA in 16 petri dishes; eight dishes each contained six mature yellow females, and eight dishes each contained six brown cysts. Half of the mature females plus eggs and half of the cysts plus eggs were inoculated with a spore and (or) hyphae suspension in sterile distilled water (0.1 ml/petri dish), and the other half of each group was inoculated with sterile distilled water as a control. The petri dishes were then placed at 25 C for 1 week.

Following the incubation period, 16 egg suspensions were prepared (one suspension per petri dish). To produce an egg suspension, the six cysts or mature females and associated eggs from a petri dish were picked up with forceps and placed in water in a hand-held homogenizer. The females

TABLE 1. Designations and origins of the fungi bioassayed.

| Fungus | Beltsville Nematology Lab designation | Source† | ATCC designation |
|---|---|---|---|
| *Acremonium bacillisporum* | 56 | PR | |
| *Chaetomium* sp. | 50 | HGG | |
| *Drechmeria coniospora* | | | |
| strain 1 | 54 | ATCC | 18767 |
| strain 2 | 55 | ATCC | 48243 |
| *Epicoccum* sp. | 2 | HGG | |
| *Exophiala jeanselmei* | 12 | HGF | |
| *Fusarium* sp. | 35 | HGF | |
| *Neocosmospora vasinfecta* | 14 | HGF | 62828 |
| *Phoma chrysanthemicola* | 20 | HGF | |
| *Scytalidium fulvum* | 9 | HGF | |
| *Trichoderma harzianum* | | | |
| strain 1 | 60 | ATCC | 24274 |
| strain 2 | 61 | ATCC | 52444 |
| *Trichoderma polysporum* | 59 | ATCC | 60004 |
| Unidentified fungus | 13 | HGF | |
| *Verticillium chlamydosporium* | | | |
| strain 1 | 62 | ATCC | 52033 |
| strain 2 | 63 | HA | |
| *Verticillium lecanii* | | | |
| strain 1 | 64 | ATCC | 46578 |
| strain 2 | 65 | ATCC | 58909 |
| strain 3 | 66‡ | HGOC | |
| strain 4 | 67§ | HGOC | |

† ATCC = American Type Culture Collection, Rockville, MD; HA = isolated from the nematode *Heterodera avenae* by D. H. Crump and B. R. Kerry, Rothamsted Experimental Station, Harpenden, UK; HGF = isolated from field-collected *Heterodera glycines* during the current study; HGG = isolated from greenhouse-collected *Heterodera glycines* during the current study; HGOC = isolated from *Heterodera glycines* by A. Ostericher Carrell, formerly at the Nematology Lab, USDA ARS, Beltsville, MD; PR = isolated from the nematode *Panagrellus redivivus* by S. L. F. Meyer during the current study.

‡ Designated 319.11 when originally isolated.

§ Designated 327.13 when originally isolated.

or cysts were disrupted to release the internal eggs, so that both internal and external eggs were suspended in water. A portion of each egg suspension was placed on a microscope slide and stained with new blue R + dimethyl sulfoxide (10). One microscope slide of egg suspension was prepared from each group of six females or cysts, making a total of 16 slides per experiment. The first 100 eggs seen on each of the 16 slides were examined at 100× or 200× magnification to determine egg viability and to see if eggs were infected by fungal hyphae. Eggs that contained live juveniles and eggs from which juveniles had hatched were counted as viable.

The bioassay was repeated with each fungus suspension and control, giving a total count of 800 eggs per treatment for each fungus; i.e., 800 inoculated eggs from mature yellow females, 800 control eggs from mature yellow females, 800 inoculated eggs from brown cysts, and 800 control eggs from brown cysts.

The experiment was analyzed as a 2 × 2 factorial with two "cyst" types (mature yellow females and brown cysts) and two "inoculation" types (water control and fungus inoculum in water). Planned comparisons were used to determine whether differences between control and inoculated were significant within a "cyst" type. Data analyzed were whole number counts. The percentages of viable eggs from each treatment were also determined.

## RESULTS

The greatest numbers of fungal isolates were obtained from field-collected cysts and eggs incubated at 25 C on V-8 (9 isolates)

TABLE 2. Percentage of live *Heterodera glycines* eggs from each bioassay treatment.

| Fungus | Inoculated eggs from mature yellow females | Control eggs from mature yellow females | Inoculated eggs from brown cysts | Control eggs from brown cysts |
|---|---|---|---|---|
| *Acremonium bacillisporum* | 89.7 | 89.2 | 93.7 | 94.2 |
| *Chaetomium* sp. | 91.7 | 89.9 | 84.5 | 90.5 |
| *Drechmeria coniospora* strain 1 | 93.1 | 94.6 | 92.6 | 96.1 |
| *Drechmeria coniospora* strain 2 | 92.9 | 94.7 | 89.2 | 90.4 |
| *Epicoccum* sp. | 92.1 | 88.0 | 89.6 | 88.7 |
| *Exophiala jeanselmei* | 91.9 | 89.2 | 92.9 | 92.9 |
| *Fusarium* sp. | 90.6 | 94.6 | 94.0 | 95.2 |
| *Neocosmospora vasinfecta* | 91.4 | 92.2 | 88.1 | 91.6 |
| *Phoma chrysanthemicola* | 87.7† | 94.5 | 97.6 | 94.4 |
| *Scytalidium fulvum* | 88.0 | 88.7 | 94.1 | 93.2 |
| *Trichoderma harzianum* strain 1 | 91.7 | 90.1 | 90.6 | 92.4 |
| *Trichoderma harzianum* strain 2 | 94.5 | 91.2 | 92.6 | 94.9 |
| *Trichoderma polysporum* | 91.6‡ | 84.7 | 94.1 | 93.7 |
| Unidentified fungus | 85.9 | 90.5 | 95.1 | 95.4 |
| *Verticillium chlamydosporium* strain 1 | 86.5 | 90.6 | 97.7 | 96.5 |
| *Verticillium chlamydosporium* strain 2 | 90.5 | 89.7 | 88.1‡ | 92.9 |
| *Verticillium lecanii* strain 1 | 95.9 | 95.6 | 91.4 | 94.2 |
| *Verticillium lecanii* strain 2 | 92.2‡ | 96.5 | 94.5 | 94.1 |
| *Verticillium lecanii* strain 3 | 92.0 | 91.1 | 89.4 | 88.5 |
| *Verticillium lecanii* strain 4 | 91.4 | 92.7 | 89.6 | 90.9 |

Live eggs included those that had hatched and those that contained live juveniles. For each fungus, the percentage of live eggs per treatment was calculated from the combined results of two bioassays, with four replicates of each treatment per bioassay. A total of 800 eggs was counted for each treatment.

† Total number of live inoculated eggs and total number of live control eggs were statistically different ($P < 0.01$) according to a single degree of freedom $F$-test.

‡ Total number of live inoculated eggs and total number of live control eggs were statistically different ($P < 0.05$) according to a single degree of freedom $F$-test.

and on WA + strep (10 isolates). The most isolates obtained from any other agar-temperature combination was three. The eight bioassayed fungi were isolated from the following media: *Chaetomium* sp. from PDA; *Epicoccum* sp. from WA + strep; *Exophiala jeanselmei* (Langeron) McGinnis & Padhye from PYG; *Fusarium* sp. from WA; *Neocosmospora vasinfecta* E. F. Smith from chitin agar, CMA, PYG, and WA + strep; *Phoma chrysanthemicola* Hollós from CMA; *Scytalidium fulvum* Morgan-Jones & Gintis from V-8 and WA; and the unidentified fungus from PYG.

Three fungi were measurably deleterious to the eggs (Table 2). Eggs from mature yellow females inoculated with *P. chrysanthemicola* or with *Verticillium lecanii* (A. Zimmermann) Viégas strain 2 had a lower ($P < 0.01$, $P < 0.05$, respectively) survival rate than uninoculated eggs (Table 2). *Verticillium chlamydosporium* Goddard strain 2 caused a decrease ($P < 0.05$) in the number of viable eggs from brown inoculated cysts as compared with viable eggs from brown control cysts.

Conversely, inoculation with *Trichoderma polysporum* (Link) Rifai resulted in an increase ($P < 0.05$) in the number of eggs from mature yellow females that survived (Table 2). When this fungus was present, more inoculated eggs than uninoculated eggs were viable.

Hyphae of *T. polysporum* were observed

in one live egg from a yellow female and in one live egg from a brown cyst. *Verticillium lecanii* strain 2 was seen in a hatched juvenile but not in a live egg. Neither *P. chrysanthemicola* nor *V. chlamydosporium* strain 2 were observed in live eggs. Hyphae of all four fungi were seen in dead eggs. The numbers of dead eggs from the treatments inoculated with fungus suspensions of these strains were as follows: *T. polysporum* colonized 6 of the 66 dead eggs from mature females and 8 of the 46 dead eggs from brown cysts; hyphae of *V. lecanii* strain 2 were observed in 30 of the 62 dead eggs from yellow females and in 11 of the 44 dead eggs from brown cysts; *P. chrysanthemicola* colonized 8 of the 98 dead eggs from mature females and 12 of the 19 dead eggs from brown cysts; hyphae of *V. chlamydosporium* strain 2 were observed in 14 of the 76 dead eggs from yellow females and in 25 of the 95 dead eggs from brown cysts.

Sixteen of the fungi tested had no measurable effect on egg survival (Table 2), even though five of them infected live eggs. Hyphae of *Drechmeria coniospora* (Drechsler) W. Gams & Jansson strain 1 and of *Scytalidium fulvum* were seen in live eggs from mature yellow females. The unidentified fungus colonized a live egg from a brown cyst. *Acremonium bacillisporum* (Onions & Barron) W. Gams and *Verticillium lecanii* strain 3 colonized live eggs from both yellow females and brown cysts.

*Trichoderma harzianum* Rifai strain 1 did not colonize dead eggs. Hyphae of *T. harzianum* strain 2 were seen in dead eggs from brown cysts. Hyphae of the other fungi were observed in dead eggs from both yellow females and brown cysts.

## Discussion

*Neocosmospora vasinfecta* and *Scytalidium fulvum* have been isolated from SCN in other areas of the United States (1,16). The strains of *Chaetomium, Epicoccum,* and *Fusarium* isolated and bioassayed were not identified to species, but one or more members of all three genera have been found associated with this nematode (1,16). Species of *Acremonium* and *Phoma,* and at least one species of *Exophiala,* have been isolated from SCN (1,16). However, *Exophiala jeanselmei* is here reported for the first time in the United States from *H. glycines. Phoma chrysanthemicola* has not previously been reported as an *H. glycines*-associated fungus nor as a plant pathogen in the United States (1,2,16). *Acremonium bacillisporum,* which was isolated from the free-living nematode *P. redivivus* during this study, has not been reported from SCN (1,16).

*Drechmeria, Trichoderma,* and *Verticillium* species were studied because strains of these fungi have shown potential as biocontrol agents against nematodes or other organisms. *Drechmeria coniospora* is a successful antagonist of several nematodes; for example, this fungus reduces galling caused by *Meloidogyne incognita* (Kofoid & White) Chitwood (7). Strain ATCC 18767 was originally isolated from a nematode by G. L. Barron (8). Species of *Trichoderma* are known to act as biocontrol agents (15), and *T. harzianum* and *T. koningii* Oudemans have been isolated from *H. glycines* in the United States (1,16). Similarly, *V. chlamydosporium* and *V. lecanii* have been successful in biocontrol studies and have been found associated with SCN (16).

Pathogenicity to nematodes may vary among strains of a species, as was illustrated in a study (6) of six strains of *V. chlamydosporium* on cyst nematode eggs. Consequently, more than one strain was tested from four of the species assayed in the current study.

*Phoma chrysanthemicola,* one strain of *V. chlamydosporium,* and one strain of *V. lecanii* were deleterious to *H. glycines* eggs, even though hyphae of these fungi were not observed in live eggs. Whether infected dead eggs had been colonized before or after death could not be determined, but the absence of live parasitized eggs and the high numbers of dead uncolonized eggs indicated that the three fungi could affect egg viability through some method other than parasitism. Previous studies have demonstrated that *Phoma* spp. cannot physically disrupt heteroderid nematode eggs, but they do appear to produce metabolites that

affect the eggs (16). In contrast, species in *Verticillium* section Prostrata, including *V. chlamydosporium* and *V. lecanii*, parasitize cysts and (or) eggs (16). However, while *V. chlamydosporium* colonized viable eggs of *H. avenae* Wollenweber and *H. schachtii* Schmidt, dead and immature eggs were most readily infected (6).

The three fungi that decreased egg viability did not kill large numbers of eggs. This result was expected with this assay method. Many juveniles hatched before the fungi grew and interacted with the eggs. Since the bioassay method does not require large amounts of previously established hyphae and spores, it should identify highly pathogenic fungi.

*Trichoderma polysporum*, though beneficial to eggs from mature yellow females, infected a live egg from a mature female. The ability to parasitize live eggs does not mean that a fungus is highly pathogenic to a population of eggs. The fungi that were isolated from nematodes but not deleterious to SCN eggs may be saprophytes on dead matter or parasites of other nematode species, or they may require specific conditions for parasitism to occur.

This bioassay method has several advantages. It employs culture-grown eggs that eliminate the problem of contamination inherent with field-collected or greenhouse-grown eggs and cysts. As in most studies of cyst nematode biocontrol, emphasis is placed on eggs because they are readily available for infection in the soil. The bioassay provides results rapidly, and also helps determine whether a fungus parasitizes live eggs or primarily affects eggs through some other mechanism.

These studies identified fungi that affect the viability of soybean cyst nematode eggs in vitro. Fungi that demonstrated antagonism to the nematode will be studied for ability to act as biocontrol agents in the soil.

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