REPRODUCTION OF *HETERODERA GLYCINES* ON SOYBEAN IN NONSTERILE SOIL INFESTED WITH CYST-COLONIZING FUNGI†

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ABSTRACT

Stiles, C. M., D. A. Glawe, G. R. Noel, and J. K. Pataky. 1993. Reproduction of *Heterodera glycines* on soybean in nonsterile soil infested with cyst-colonizing fungi. Nematropica 23:81–89.

Six fungi capable of colonizing roots of soybean were isolated from cysts of *Heterodera glycines* recovered from field soil suppressive to the nematode. The fungi were evaluated for the ability to reduce reproduction of first-generation *H. glycines* under greenhouse conditions. *Diheterospora chlamydosporia* (Synonym: *Verticillium chlamydosporium*), *Fusarium oxysporum, F. solani, Paraphoma radicina, Pyrenochaeta terrestris,* and *Stagonospora heteroderae* were inoculated separately onto roots of 3- and 10-day-old 'Williams 82' soybean seedlings that were transplanted into nonsterile soil free of *H. glycines*. Second-stage juveniles (J2) of *H. glycines* were added 3 days after inoculating with fungi and numbers of first-generation cysts, eggs, and J2 were determined. The fungi did not reduce either the number of cysts or the total number of eggs and juveniles. Inoculation of roots with *F. oxysporum* resulted in a significant ($P = 0.10$) increase in numbers of cysts and of eggs and juveniles when compared to the control. None of the fungi was isolated from the yellow female stage, but all of the fungi except *D. chlamydosporia* were isolated from cream to brown colored cysts. All fungi were isolated from roots to which they were inoculated.

Key words: biological control, *Diheterospora chlamydosporia, Fusarium oxysporum, Fusarium solani, Glycine max, Heterodera glycines, Paraphoma radicina, Pyrenochaeta terrestris,* soybean, *Stagonospora heteroderae.*

RESUMEN

Stiles, C. M., D. A. Glawe, G. R. Noel y J. K. Pataky. 1993. Reproducción de *Heterodera glycines* en soya en suelo no esterilizado infestado con hongos colonizadores de quistes. Nematrópica 23:81–89.

Seis hongos capaces de colonizar raíces de soya fueron aislados de quistes de *Heterodera glycines* obtenidos a partir de suelo de campo supresor del nematodo. Se evaluó la habilidad de los hongos para reducir la reproducción de la primera generación de *H. glycines* bajo condiciones de invernadero. *Diheterospora chlamydosporia* (Sinónimo: *Verticillium chlamydosporium*), *Fusarium oxysporum, F. solani, Paraphoma radicina, Pyrenochaeta terrestris* y *Stagonospora heteroderae* fueron inoculados por separado en las raíces de plántulas de soya 'Williams 82' de 3 y 10 días de edad que fueron transplantadas en suelo no esterilizado libre de *H. glycines*. Se añadieron juveniles del segundo estadío (J2) de *H. glycines* 3 días después de inocular los hongos y se determinó el número de quistes de la primera generación, huevos y juveniles. Los hongos no redujeron el número de quistes así como tampoco el número total de huevos y juveniles. La inoculación de las raíces con *F. oxysporum* dió como resultado un incremento significativo ($P = 0.10$) en el número de quistes, huevos y juveniles en comparación con el control. Ninguno de los hongos fue aislado a partir del estadío de hembra amarilla, pero todos los hongos, excepto *D. chlamydosporia* fueron aislados de quistes cuyo color variaba entre crema y marrón. Todos los hongos fueron aislados a partir de las raíces en las que fueron inoculados.

Palabras clave: control biológico, *Diheterospora chlamydosporia, Fusarium oxysporum, Fusarium solani, Glycine max, Heterodera glycines, Paraphoma radicina, Pyrenochaeta terrestris,* soya, *Stagonospora heteroderae.*

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INTRODUCTION

The soybean cyst nematode, *Heterodera glycines* Ichinohe, is a serious pest of soybean [*Glycine max* (L.) Merr.]. Impetus for research on biological control of the nematode was provided by the discovery that naturally occurring, soilborne fungi suppress populations of the cereal cyst nematode, *Heterodera avenae* Woll. (15). Subsequent studies have shown that numerous fungi colonize cysts of *H. glycines* in the United States (2,3,11,12,18-20).

Certain cyst-colonizing fungi are able to destroy eggs and juveniles of *H. glycines* (13). However, little is known concerning how cyst-colonizing fungi interfere with the reproduction and feeding of *H. glycines* on soybean roots. Given the importance of endophytic fungi in reducing insect damage to infected plants (27), it seems reasonable that root-colonizing fungi could afford soybean a degree of protection from *H. glycines* if similar mechanisms were operative. Research on other combinations of nematodes, fungi, and host plants has shown that nematodes can be affected by fungi present in the host plant. A soybean isolate of *Fusarium oxysporum* Schlect. invaded giant cells in soybean roots infected by *Meloidogyne incognita* (Kofoid & White) Chitwood, resulting in an increase in the ratio of males to females (21). Ultrastructural changes of syncytia induced by *Meloidogyne javanica* (Treub) Chitwood in tomato (*Lycopersicon esculentum* Mill.) indicate that giant cells may be disrupted by a fungal metabolite of *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. & Hans. (10), and a number of other reports also have documented fungal interference with nematode syncytia and giant cells (1,8,25). Crump (5) suggested that a fungus such as *Cylindrocarpon destructans* (Zins.) Scholten, which colonizes the cortex of sugar beet (*Beta vulgaris* L.) roots, might interfere with the development of giant cells.

Although fungi isolated from cysts of *H. glycines* can colonize soybean roots (3, 30), no published studies have examined the possible effect of these fungi on reproduction of the nematode after inoculating soybean roots with the fungi. The experiments reported herein used cyst-colonizing fungi obtained from field soil suppressive to *H. glycines* to test whether they can interfere with production of cysts and reproduction of *H. glycines* in nonsterile field soil.

MATERIALS AND METHODS

Isolates of *Diheterospora chlamydosporia* (Goddard) Barron & Onions (Synonym: *Verticillium chlamydosporium* Goddard), *Paraphoma radicina* (McAlp.) Morgan-Jones & White, *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson, *Stagonospora heteroderae* Carris, Glawe & Morgan-Jones, *Fusarium solani* (Mart.) Appel & Wollenw., and *Fusarium oxysporum* Schlect. isolated previously from cysts of *H. glycines* (4) were grown separately in 50 ml of glucose mineral medium (29) in glass bottles for approximately 1 week. Mycelia of each fungus were removed and placed in a sterilized tissue homogenizer. Approximately 1.5 ml of sterile deionized water was added and the tissue was ground and poured into a 250-ml flask containing 50 ml sterilized glucose mineral medium. Flasks were placed on a shaker (ca. 25 °C). After 2 days, mycelia of each isolate were collected on an 8-μm filter and rinsed three times in sterile, deionized water. Mycelia were mixed with water in a Waring blender and were poured into a flask with additional water to produce a concentration of 2 g of fungal tissue per 100 ml of suspension. The number of colony-forming units (CFU) per ml of suspension

were determined by serial dilution (Table 1). The concentrations of fungal inoculum suspensions ranged from 1.72×10^4 to 5.99×10^6 CFU/ml (Table 1). The number of propagules per ml were higher for the hyphomycetous fungi (*D. chlamydosporia, F. solani,* and *F. oxysporum*) than for the pycnidial fungi (*Paraphoma radicina, Pyrenochaeta terrestris,* and *S. heteroderae*), although on a weight basis inoculum levels were equal. Inoculum suspensions of the hyphomycetous fungi contained conidia, mycelial fragments, and in some cases chlamydospores, whereas the pycnidial fungi did not produce conidia in liquid shake cultures.

For the first and second experiments, soybean seeds (cv. Williams 82) were germinated as described by McClure and Robertson (17) and seedlings with radicles 4–6 cm long (3 days old) were used. In the third experiment, soybean seeds were germinated in sand in the greenhouse. In each case, roots of each seedling were dipped in inoculum suspension for 30 s (3-day-old seedlings, experiments 1 and 2) or 2 min (10-day-old seedlings, experiment 3), then transplanted to nonsterile, *H. glycines* free soil [series Watseka (sandy, mixed, mesic Aquic Hapludolls); surface layer texture = loamy fine sand] in a 7-cm-diam clay pot. Nonsterile soil was used to allow natural mycoflora to compete with the inoculated fungi. Either 50 ml of inoculum suspension (1 g of fungal tissue) or, for the control treatment, sterile deionized water was poured around the seedling. A 1-cm-diam test tube was placed into the soil in each pot *ca.* 2 cm from the seedling to form a 1-cm-deep well for subsequent nematode inoculation. After fungal inoculation, a thin layer of soil was sprinkled on the soil surface and moistened.

Table 1. Colony-forming units (CFU) in 0.02 g/ml suspensions of fungal inoculum.

Fungus	CFU/ml[z]
Paraphoma radicina	5.86×10^3
Pyrenochaeta terrestris	2.46×10^4
Stagonospora heteroderae	5.27×10^5
Diheterospora chlamydosporia	4.06×10^6
Fusarium solani	1.10×10^6
Fusarium oxysporum	5.99×10^6

[z]Means of five inoculum suspensions per fungus except *P. radicina,* which was based on four suspensions.

A population of *H. glycines* race 3 from a soybean field near Sidney, Champaign County, Illinois, was maintained on soybean (cv. Williams 82) in a greenhouse. Cysts were obtained from these cultures by wet-screen sieving (28) followed by centrifugation in tubes containing a 25% sucrose solution layered onto a 50% sucrose solution. The tubes were centrifuged until 800 *g* was obtained (*ca.* 2 min). Cysts were removed from the interface of the two sucrose solutions, rinsed thoroughly with tap water, and then crushed in a tissue grinder. Crushed cysts were placed on Baermann funnels in a mist chamber and second-stage juveniles (J2) were collected in test tubes. Alternatively, J2 were obtained either from intact or crushed cysts placed on modified Baermann funnels placed on a laboratory bench.

In the third experiment, additional 10-day-old seedlings were selected randomly to determine whether fungi were present in the roots and cysts at the time of inoculation. Roots were dipped in the fungal inoculum suspensions, surface-sterilized in 2.6% $NaHClO_4$ for 30 s, and placed on 1.5% water agar plates amended with streptomycin sulfate (100 μg/ml). Also in this experiment, samples of intact cysts extracted from soil and samples of cysts from Baermann funnels were surface-sterilized in 0.5% $NaHClO_4$ in a Millipore filter system for 3 min, rinsed

three times with sterile deioized water, and placed on the same medium.

Three days after seedlings were inoculated with fungi, 800 J2 were introduced into the infestation well in each pot. Treatments were replicated over time and maintained in a greenhouse in a randomized complete block design. There were four, three, and four replications in the first, second, and third experiments, respectively. Each replicate consisted of two complete sets of fungal and control treatments and additional control pots.

When first-generation females had formed on roots of plants (32–40 days after nematode infestation), females and cysts were extracted from one set of pots by wet-screen sieving as described above and removed from the screenings using a watchmaker's forceps. Cysts were crushed in a tissue grinder to free eggs and juveniles. The number of eggs and juveniles was determined by diluting the suspension to a known volume, counting three 1-ml aliquants, and calculating the total number of eggs and juveniles per plant. Fresh and dry weights of both roots and shoots were obtained.

Plants were removed from the second set of pots to determine whether fungi had colonized roots and cysts. Tissue samples (3–8 mm long) were cut from the primary root of each plant, surface sterilized and placed on agar medium as described for isolations from 10-day-old seedlings. In the first and second experiment, 20 yellow females from each treatment were surface sterilized and placed on agar medium as described. In the third experiment, yellow females and cream to brown colored cysts were surface sterilized and placed separately on agar plates. Fungal colonies were transferred to cornmeal agar plates for identification. Additional cysts were removed from the screenings, crushed under coverslips, and examined microscopically for signs of fungi (hyphae and/or resting structures). Number of cysts, number of eggs and juveniles per plant, and number of eggs and juveniles per cyst were $\log_{10}$ transformed to standardize variance and were analyzed by ANOVA. Treatment means were compared by FLSD values ($P = 0.10$).

RESULTS AND DISCUSSION

No fungi were recovered from roots of uninoculated 10-day-old soybean seedlings at the time of inoculation, indicating that the seedlings used were free of fungi prior to initiating the experiment. One of the test fungi, *D. chlamydosporia*, was recovered from one inoculated root immediately following inoculation, suggesting that it had escaped the effects of surface sterilization. No fungal growth occurred from the 105 intact egg-producing females plated at the time of nematode infestation, indicating that the yellow females used to obtain J2 were free of culturable fungi.

Conidia produced by the hyphomycetous fungi in liquid shake cultures, in addition to mycelia and chlamydospores, may have led to the higher numbers of CFU in the inoculum suspensions than those of the pycnidia-forming fungi. In addition, different types of propagules might vary in their ability to germinate and cause infections, and thus they may not be equally effective as inoculum. Kerry *et al.* (16) noted that an isolate of *Verticillium chlamydosporium* Goddard (*D. chlamydosporia*) which produced a larger number of propagules than other isolates also caused the greatest decrease in reproduction of *H. avenae* on wheat. In spite of possibly large differences among the numbers of propagules introduced, five of the six fungi (all except *D. chlamydosporia*) were recovered from cream to

brown colored cysts and none of the inoculated fungi was recovered from yellow females (Table 2). Each of the test fungi was recovered from roots of soybean plants grown in soil to which the fungus had been introduced (Table 2). *Pyrenochaeta terrestris* also was recovered from one cream to brown colored cyst and one soybean root from the control treatment. Additional fungi that were recovered occassionally from roots included common soil inhabitants, such as species of *Fusarium* and *Chaetomium*. These fungi were recovered from 6.7% (12 of 179) of the yellow females and 69% (20 of 29) of the cream to brown colored cysts from the sterile water control treatment. Cysts examined microscopically that had fungi (hyphae and/or resting structures) associated with eggs and juveniles inside cysts ranged from 7.3 to 21.5% (Table 3) (n = 103–164 cysts observed per treatment). Hyphae associated either with the egg mass or the outside of the cyst ranged from 25.8 to 42.3%. Even though fungi were frequently present in cysts, eggs and juveniles usually appeared healthy.

Analysis of root-to-shoot weight ratios by ANOVA and LSD did not reveal any consistent differences due to treatments (data not shown), and no discoloration or lesions were observed on inoculated roots. Thus, the test fungi apparently did not cause plant disease. In an earlier study, Stiles and Glawe (30) found that fungi isolated from cysts of *H. glycines* could colonize roots of soybean seedlings without causing apparent disease.

Nematode reproduction data from the three experiments were combined (Table 4). The mean number of cysts per plant ranged from 57.4 to 88.6, the mean number of eggs and juveniles per plant ranged from 12 100 to 21 200, and the mean number of eggs and juveniles per cyst ranged from 192 to 254 (Table 4). Analysis of untransformed and $\log_{10}$ transformed data by ANOVA and separation of means using the least significant difference ($P = 0.10$) revealed few differences among treatments for any of these parameters. Increasing evidence suggests that specialized biotypes of nematode-suppressive fungi may exist (7,14,23), and some isolates of cyst-infecting fungi may differ in how they affect nematode reproduction. However, greater numbers of cysts per plant, and eggs and juveniles

Table 2. Percentage of surface-sterilized first-generation cysts of *Heterodera glycines* and percentage of soybean roots, from which test fungi were recovered 32 to 40 days after inoculation of soybean seedlings (cv. Williams 82) with fungi and second-stage juveniles of *H. glycines*.

Fungal treatment	Experiments 1 & 2[x]		Experiment 3[y]		
	Yellow females[z] (n = 138–140)	Roots (n = 7)	Yellow females (n = 29–44)	Cream-brown cysts (n = 28–47)	Roots (n = 4)
Diheterospora chlamydosporia	0	0	0	0	50
Pyrenochaeta terrestris	0	43	0	15	50
Paraphoma radicina	0	29	0	21	75
Stagonospora heteroderae	0	14	0	13	75
Fusarium solani	0	43	0	8	25
Fusarium oxysporum	0	43	0	11	50

[x]Two experiments combined, seven replicates.
[y]One experiment, four replicates.
[z]n = number of females, cysts, or roots examined per treatment.

Table 3. Percentages of first-generation cysts with and without signs of fungi (hyphae and/or resting structures) after inoculation of 'Williams 82' soybean seedlings with fungi and second-stage juveniles of *Heterodera glycines*.

Treatment[z]	No hyphae apparent	Hyphae found only within egg mass or outside cyst	Hyphae present inside cyst
Sterile H_2O	45.4	41.1	12.8
Diheterospora chlamydosporia	50.4	28.1	21.5
Pyrenochaeta terrestris	47.2	42.3	10.6
Paraphoma radicina	59.1	25.8	15.1
Stagonospora heteroderae	59.1	33.5	7.3
Fusarium solani	47.0	33.0	20.0
Fusarium oxysporum	58.3	28.2	13.6

[z]Three experiments combined, 11 replicates; n = 103–164 cysts observed per treatment.

per plant were recovered from roots inoculated with *F. oxysporum* than from roots of the sterile water control.

All of the test fungi colonized roots of soybeans, but because the fungi were not recovered from young females, they apparently did not infect females via syncytia in the soybean roots. Carris *et al.* (4) found that the lowest percentages of *H. glycines* cysts containing fungi occurred during the time of nematode reproduction (*i.e.*, during the growing season), suggesting that during reproduction new cysts form faster than fungi can colonize them. Thus, the high reproductive capacity of cyst nematodes (15,26) may allow populations to be maintained in field situations, even though cysts frequently may be colonized by fungi. Test fungi in the present study were recovered from older cream to brown colored cysts. The presence of fungi observed in cysts with healthy eggs and juveniles suggests that some fungi may colonize cysts without apparent effects. In the present study, to facilitate screening of fungi, only one generation

Table 4. Mean number of *Heterodera glycines* cysts, eggs and juveniles per plant after one generation of reproduction on 'Williams 82' soybean following inoculation with 800 second-stage juveniles of *H. glycines* and six fungi.

Treatment	Cysts per plant[z]	Eggs & juveniles per plant	Eggs & juveniles per cyst
Sterile H_2O	57.4	12 400	222.4
Pyrenochaeta terrestris	59.5	12 100	228.8
Stagonospora heteroderae	70.7	14 300	216.4
Diheterospora chlamydosporia	70.8	14 400	222.6
Paraphoma radicina	71.0	16 500	254.1
Fusarium solani	78.2	15 600	192.2
Fusarium oxysporum	88.6	21 200	228.5
FLSD ($P = 0.10$)	26.9	6 200	39.8
CV (%)	53.3	56.7	25.0

[z]Three experiments combined, means of 11 replicates.

of the nematode was allowed to develop. Thus, test fungi were not allowed to colonize cysts over several generations. Suppression of nematode reproduction by fungal colonization of cysts may require more than one generation to be effective. For instance, naturally-occurring suppression of *H. schachtii* Schmidt on sugar beet was most active during the second generation of reproduction (6). Gintis *et al.* (12) found a different mycota associated with young females of *H. glycines* compared to that of older cysts. Fungi might become associated with the nematode at various stages during the season, such as with the infective juvenile stage, or during the development of the reproducing female, or during the cyst stage in soil. Fungi present with these different stages of the nematode might affect reproduction by different mechanisms.

Plants inoculated with *F. oxysporum* produced a greater number of cysts per plant and a greater number of eggs and juveniles per plant (Table 3) than was the case on uninoculated controls. Similar findings have been reported by other researchers. Edmunds and Mai (9) showed that *Pratylenchus penetrans* (Cobb) Chitwood & Oteifa was attracted to and invaded more alfalfa (*Medicago sativa* L.) roots infected with *F. oxysporum* than uninfected plants. Numbers of *H. daverti* Wouts & Sturhan cysts increased on subterranean clover (*Trifolium subterraneum* L.) when plants also were inoculated with *F. avenaceum* (Fr.:Fr.) Sacc. and *F. oxysporum* (22). Significantly higher populations of *H. glycines* developed on soybean (cv. Lee) in microplots inoculated with *F. oxysporum* than in uninoculated plots (24). 'Lee' soybean was believed to have considerable tolerance to Fusarium wilt even in the presence of *H. glycines*. The increase in the nematode population was believed due to stimulation of root growth caused by the oat-*Fusarium* inoculum. In the present study, significantly greater numbers of cysts per plant, and eggs and juveniles per plant, occurred on *F. oxysporum*-inoculated plants than in the sterile water control; in this experiment no oat or other inoculum substrate was provided. The ability of *F. oxysporum* to increase cyst nematode reproduction indicates that interactions of fungi, nematodes, and host plants do not always occur at the expense of nematode reproduction. Given the ubiquitous occurrence of *F. oxysporum* in field soils, research on the mechanism by which this fungus can increase cyst nematode reproduction promises to provide significant information on the effect of root- and cyst-colonizing fungi on reproduction of nematodes in production fields.

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