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Fungal Parasitism of *Heterodera glycines* Eggs as Influenced by Egg Age and Pre-colonization of Cysts by Other Fungi[1]

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Abstract: The objective of this study was to determine the effect of egg age and pre-colonization of cysts by a saprophytic or parasitic fungus on parasitism of *Heterodera glycines* eggs by other parasitic fungi. In agar and in soil tests, fungi generally parasitized more eggs in early developmental stages than eggs containing a juvenile. The effect of pre-colonization of cysts by a fungus on parasitism of eggs by other fungi depended on the fungi involved. In most cases, pre-colonization of cysts by an unidentified, saprophytic fungal isolate (A-1-24) did not affect parasitism of eggs in the cysts subsequently treated with other fungi. However, pre-colonization of cysts by A-1-24 reduced fungal parasitism of eggs in cysts subsequently treated with *Cylindrocarpon destructans* isolate 3. In agar tests, pre-colonization of cysts by *Chaetomium cochliodes,* a saprophytic or weakly parasitic fungus, reduced parasitism of eggs in cysts subsequently treated with *Verticillium chlamydosporium* Florida isolate, *Fusarium oxysporum, Fusarium solani,* ARF18, and another sterile fungus. However, in soil tests, pre-colonization of cysts by *C. cochliodes* had no effect on parasitism of eggs by subsequent fungal parasites. In another test, parasitism of eggs by *V. chlamydosporium* in cysts was not affected by pre-colonizing fungi *C. destructans, F. oxysporum,* and *F. solani* but was reduced by *Mortierella* sp., *Pyrenochaeta terrestris,* and *C. cochliodes.* Parasitism of eggs in cysts by ARF18 was reduced by pre-colonizing fungi *C. destructans, F. oxysporum, F. solani, P. terrestris,* and *C. cochliodes* but not *Mortierella* sp.

Key words: biological control, cysts, cyst nematode, eggs, *Heterodera glycines,* nematophagous fungi, soybean cyst nematode.

Many fungi have been isolated from females, cysts, egg masses, and eggs of Heteroderidae. Some of them are able to parasitize nematode eggs. However, parasitism of nematode eggs by fungi may be affected by many factors. Some reports indicate that immature eggs are more susceptible to the fungal attack, but limited experimental evidence is available in the literature. Stirling and Mankau (1979) observed that *Meloidogyne* eggs in the early developmental stage were more susceptible to *Dactylaria oviparasitica* than eggs containing second-stage juveniles (J2). Eggs of *Heterodera schachtii* containing J2 appeared to be resistant to parasitism by *Acremonium strictum* and *Fusarium oxysporum,* although a few J2 within eggs were colonized by *A. strictum* (Nigh et al., 1980). Jatala (1986) reported that nematode eggs in early embryonic stages prior to the gastrulation process were more vulnerable to infection by *Paecilomyces lilacinus.* Two isolates of an unidentified fungus, designated as ARF18, were tested for their pathogenicity to the soybean cyst nematode, *Heterodera glycines,* on water agar (Kim and Riggs, 1991). Isolate ARF18-A infected 89% of eggs in yellow females and 61% of eggs in brown cysts, whereas isolate ARF18-B infected 51% of eggs in yellow females and 57% of eggs in brown cysts. However, the developmental stages of eggs in the females or cysts were not determined. Presumably, yellow females contained more immature eggs than the cysts. The fungus ARF18 also can colonize first-stage juveniles (J1) in eggs (Kim et al., 1992). Irving and Kerry (1986) quantified the parasitism of immature eggs and eggs containing J2 of *Heterodera avenae* by four strains of *Verticillium chlamydosporium* (=*Diheterospora chlamydosporia*). All strains parasitized more eggs that had not completed their embryonic development than eggs that contained J2.

Some fungi isolated from females, cysts, and egg masses may be saprophytes. Nematophagous fungi and saprophytic fungi may compete for nutrients in cysts and egg masses. While Hay and Skipp (1993) reported that multiple species of fungi are commonly encountered in cysts of *H. trifolii* collected from pasture soil in New Zealand, many studies (Carris et al., 1989; Chen and Chen, 2002; Chen et al., 1994; Gintis et al., 1983; Morgan-Jones and Rodríguez-Kábana, 1985) suggest that cysts of *H. glycines* colonized by one fungus are not readily colonized by other fungi. Colonization of females and cysts by saprophytic fungi may inhibit egg-parasitic fungi and therefore protect nematode eggs from being destroyed by nematophagous fungi.

The objectives of this study were to determine (i) the effects of egg age on fungal parasitism of *H. glycines* eggs and (ii) the effects of pre-colonization of females and cysts by a saprophytic or parasitic fungus on the infection of eggs by other parasitic fungi.

Materials and Methods

Effects of egg age on fungal parasitism: Test 1. Cysts of *H. glycines,* race 3, were collected from a soybean field at the University of Minnesota Southern Research and Outreach Center at Waseca, Minnesota, in October 1995. The cysts were stored in a solution containing 100 mg of streptomycin, 50 mg of chlortetracycline, and 30 mg of quinolinol per liter in a refrigerator until used in February 1996. Eggs were released from the cysts by a modified mechanical method (Niblack et al., 1993) and separated from debris by centrifugation in 35% (w/v) sucrose solution. The eggs were used as inoculum for

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greenhouse culture. This population of *H. glycines* was used throughout the study.

Sterile sand and field soil were mixed at 4:1 and placed in 20-cm-diam. pots (10 in number). Soybean 'Sturdy' was sown on 2 February 1996, and the pots were maintained in the greenhouse. After 3 days, each pot received 15,000 *H. glycines* eggs. Fifty days after inoculation, soybean roots with rhizosphere soil were removed and carefully washed with tap water. Females were dislodged from the roots with a vigorously applied water stream and washed through an 850-µm-aperture sieve onto a 250-µm-aperture sieve. The females and cysts also were recovered from the soil by decanting soil suspension through an 850-µm-aperture sieve onto a 250-µm-aperture sieve. The material on the 250-µm-aperture sieve was centrifuged in a discontinuous gradient of 10%, 30%, 50%, 70% (w/v) sucrose solutions. White to yellow females were handpicked from the 30% sucrose fraction. Brown cysts were handpicked from the 50% sucrose fraction, which contained most of the brown cysts from the culture, and from the 30% sucrose fraction. Of the eggs in the females, 64% contained embryos, 18% contained J1, and 18% contained J2. Of the eggs in the cysts, 1.7% contained embryos, 4.6% contained J1, and 93.7% contained J2. The females and cysts were surface-disinfected with 0.5% NaOCl for 3 minutes and placed on water agar. The females and cysts that showed no signs of fungal growth on water agar after 3 days were used. They were placed in a beaker, passed through an 850-µm-aperture sieve onto a 250-µm-aperture sieve to remove agar, and then treated overnight with a solution of 100 mg streptomycin plus 50 mg chlortetracycline per liter water.

The experiment was a factorial design including treatments of nematode egg age and fungi. Fourteen fungal isolates belonging to eight species were tested: four isolates of *Cylindrocarpon destructans;* two isolates each of *F. oxysporum, Gliocladium catenulatum,* and *V. chlamydosporium;* and one isolate each of *F. solani, Phialophora* sp., *Phoma* sp., and an unidentified fungus (isolate A-1-24). Except for one isolate of *V. chlamydosporium* that was from Florida (Chen et al., 1996a), all others were isolated in 1996 from *H. glycines* cysts in the same field where the nematodes were collected. The selected fungi were frequently encountered in *H. glycines* cysts in the field (S. Chen, unpubl.). *Verticillium chlamydosporium* has been previously tested on *H. glycines* eggs in light-brown to brown cysts and showed a high pathogenicity to the *H. glycines* eggs (Chen et al., 1996b).

Blocks (1 cm × 1 cm) of 2 to 3-week-old fungal cultures on cornmeal agar were transferred to 1.5% water agar in 10-cm-diam. petri dishes. Twelve to 15 fungus-free females or cysts were placed on water agar next to fungal blocks in each petri dish. Each fungal isolate was replicated three times (three petri dishes per isolate for females and three petri dishes per isolate for cysts), and one group of females or cysts was plated on water agar without fungal culture to serve as an untreated control.

After 3 weeks at room temperature (20–24 °C), the females and cysts were removed, placed on slides, covered with a glass coverslip, ruptured by pressing the coverslip, and observed with the aid of a light microscope at ×100–200 magnification. An egg-parasitic index (EPI) for each fungus was recorded based on the following 0-to-10 scale: 0 = no eggs colonized, 1 = 1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, and 10 = 91–100% eggs colonized (Chen et al., 1996b). The percentage of eggs colonized by fungus was determined by estimating rather than by counting eggs one by one. This procedure allows us to quantify a large number of samples within a short period of time. Colonization of eggs by a fungus does not mean the eggs were parasitized. However, we assume that a higher EPI indicates higher parasitic activity. A fungus colonizing a low percentage of eggs (e.g., EPI less than 1) could be saprophytic.

Test 2. The same nematode population used in Test 1 was cultured in a tank containing sterile sand-soil mixture (4:1) in a growth room at 20–28 °C with photoperiod of 16 hours of light and 8 hours of dark. Soybean was planted at two dates to produce females and cysts of different ages. Soybean roots were removed from the tank, and females and cysts were dislodged from the roots. The females and cysts were separated from debris by centrifugation in 71% (w/v) sucrose solution. Brown cysts and white to yellow females were separately handpicked. Of the eggs in the females, 60% contained embryos, 14% contained J1, and 26% contained J2. Of the eggs in the cysts, 2% contained embryos, 2% contained J1, and 96% contained J2. Fungi used in this test included *V. chlamydosporium* (Florida isolate), *F. oxysporum, C. destructans, F. solani, Pyrenochaeta terrestris,* ARF18 (isolate TN14), and the Sterile Fungus 1. All of these fungi parasitize *H. glycines* eggs (F. Chen, unpubl.). Except for *V. chlamydosporium* and ARF18, all fungi were isolated from *H. glycines* cysts from Minnesota in 1997 (Chen and Chen, 2002). ARF18 was isolated from *H. glycines* in Tennessee and was provided by Patricia Timper, USDA-ARS, Tifton, Georgia (originally from Robert Riggs, University of Arkansas). The procedures for fungal inoculation on water agar and measurements of fungal parasitism of eggs were the same as described in Test 1.

Test 3. In this test, fungal colonization of eggs in females and cysts was examined in various soils from Minnesota, which contained natural or introduced fungal parasites. Seven soil treatments were used: *Treatment 1,* a sandy loam soil with 61% sand, 24% silt, 15% clay, 1.5% organic matter, and pH 6.9 collected from a soybean field without *H. glycines* infestation in Le Sueur County, Minnesota; *Treatment 2,* soil from Treatment 1 amended with corn-grits culture of *V. chlamydosporium*

(Florida isolate) at a rate of 0.5% w/w (dry weight of corn-grits to soil) (Chen et al., 1996b) with the resulting colony-forming unit (CFU) of 2.5×10^8/g soil; *Treatment 3,* soil from Treatment 1 amended with corn-grits culture of ARF18 at a rate of 0.5% w/w (dry weight of corn-grits to soil) with the resulting CFU of 4.9×10^4/g soil; *Treatment 4,* a sandy loam soil with 58% sand, 22% silt, 20% clay, 7% organic matter, and pH 7.7 collected from a soybean field infested with a high density of *H. glycines* in Steele County, Minnesota; *Treatment 5,* a clay loam soil with 33% sand, 37% silt, 30% clay, 6% organic matter, and pH 6.3 collected from a soybean field without *H. glycines* in Waseca County, Minnesota; *Treatment 6,* a clay loam soil with 28% sand, 37% silt, 35% clay, 6.1% organic matter, and pH 6.8 collected from an *H. glycines*-infested field, which has been in soybean monoculture for 29 years and appeared to be suppressive to SCN, in Waseca County, Minnesota; and *Treatment 7,* the same soil from Treatment 1 was autoclaved and used as a control without fungus. For each soil treatment, 10 cm^3 soil was added to each of 16 50-ml conical centrifuge tubes; eight tubes of each soil received 30 females, and the other eight tubes received 30 cysts. The females or cysts, from the same batches used in Test 2, were mixed thoroughly with the soil and incubated at room temperature (22–24 °C) for 3 weeks. The females or cysts were extracted from the soils, and fungal parasitism was determined following the procedures described previously.

Effects of pre-colonization of cysts on fungal parasitism of eggs by other fungi: Test 4. In this test, the effects of pre-colonization of cysts by a saprophytic fungus on fungal parasitism of eggs by other fungi were examined on cornmeal agar. The parasitic fungi as post-colonizers of cysts used in this experiment included two isolates each of *C. destructans* and *V. chlamydosporium,* and one isolate each of *F. oxysporum, G. catenulatum,* and *F. solani,* which also were used in Test 1. A total of approximately 400 cysts (from the same batch of females used in Test 1) were pre-colonized by the unidentified fungal isolate A-1-24, which apparently is not a parasite of eggs, by the following methods. The cysts of *H. glycines* were placed on the culture of A-1-24 on cornmeal agar for 4 days. Another 400 cysts were placed on cornmeal agar without fungus for the same period of time. After the 4 days, 35 to 45 pre-colonized cysts or fungus-free cysts were exposed to each of the post-colonizing, parasitic fungi in three petri dishes (three replicates) or the cysts were placed on agar without fungus as control. The EPI were recorded following the procedures as described in Test 1.

Test 5. This test was similar to Test 4, but the parasitic post-colonizers were the same isolates used in Test 2. The cysts were from the same batch of females used in Test 2. The cysts were pre-colonized by an isolate of *Chaetomium cochliodes* (a non-parasite or weak parasite of *H. glycines* eggs) or used without pre-colonization. All other procedures were the same as in Test 4.

Test 6. In this test, the effect of fungal pre-colonization of cysts by a saprophytic or weakly parasitic fungus on parasitism of eggs subsequently exposed to soil containing parasitic fungi was determined. Cysts were either pre-colonized by *C. cochliodes* or not pre-colonized. The seven soil treatments in Test 3 were used in this test. For each treatment, 30 cysts were added in each of eight conical tubes (eight replicates). Other procedures for incubation of the cysts in the soils were the same as in Test 3.

Test 7. This test was designed to determine the effect of pre-colonization of cysts by weak to moderate parasites and non-parasites on parasitism of eggs by subsequently inoculated parasitic fungi on agar. The cysts used in this test were from the same batch of females in Test 2. The pre-colonizing fungi included the weak to moderate parasites *F. oxysporum, C. destructans,* and *P. terrestris,* and two species of saprophytic or weakly parasitic fungi *C. cochliodes* and *Mortierella* sp., which were isolated from *H. glycines* cysts from Minnesota fields (Chen and Chen, 2002). Cysts without fungal pre-colonization were included as a control. The cysts pre-colonized with fungi and the cysts without pre-colonization were subsequently exposed to either of the two highly pathogenic fungi, *V. chlamydosporium* or ARF18, on water agar or placed on the agar without fungus. Fungal parasitism was determined after 3 weeks as described previously.

Test 8. In this test, the effect of pre-colonization of cysts by weak to moderate parasites and non-parasites on subsequent parasitism of eggs by the two parasitic fungi, *V. chlamydosporium* and ARF18, was determined in soil. A soil colonized by *V. chlamydosporium* (Soil Treatment 2) and a soil colonized by ARF18 (Soil Treatment 3) were prepared with the procedures used in Test 3. A sterile soil was included as a control. Cysts pre-colonized by the same fungi as in Test 7 and cysts without pre-colonization were used. The cysts were added to the soils, and after 3 weeks they were recovered from the soil and fungal parasitism of eggs was determined.

Statistical analyses: The data were subjected to two-way analysis of variance (ANOVA). Means were compared with the least significant difference (LSD) test. For Tests 7 and 8, a three-way ANOVA was conducted to determine whether there was an interaction between the effect of pre-colonization and medium (agar vs. soil). Both tests were conducted at the same time and differed only with respect to incubation on agar or in soil.

Results

Effects of egg age on fungal parasitism of eggs: Tests 1 and 2. Parasitism of *H. glycines* eggs on agar varied among

fungi and was affected by egg age (Table 1). There was an interaction between fungal treatment and egg age ($P < 0.001$), indicating that the effect of egg age on parasitism varied among fungi. The Florida isolate of *V. chlamydosporium* parasitized the highest percentage of eggs in females and was capable of colonizing juveniles in eggs from cysts. The fungus ARF18 and Sterile Fungus 1 also had a high EPI (6.6 and 7.9, respectively) in females. The Sterile Fungus 1 parasitized the highest percentage of eggs in cysts. *Cylindrocarpon destructans, F. oxysporum, Phoma* sp., *F. solani, P. terrestris,* and *V. chlamydosporium* Minnesota isolate were moderately parasitic to eggs in the females. Most of these fungi appeared to be non-parasitic to eggs containing J2 from cysts. *Cylindrocarpon destructans* isolate 5, however, showed an EPI of 2.1 in the cysts, suggesting it might colonize some J2 in eggs. *Gliocladium catenulatum, Phialophora* sp., and the fungus A-1-24 exhibited a low EPI in both females and cysts, and they may represent weakly parasitic or non-parasitic fungi. Colonization of a small portion of eggs was also observed in the cysts and females that were not exposed to the test fungi (control) in Test 2.

Test 3. Effect of egg age on fungal parasitism in soil was similar as on agar in most cases. Higher parasitism of eggs was observed in females than in cysts in three soil treatments (Treatments 1, 2, and 4) (Table 2). No difference in egg parasitism was observed between the females and cysts in two soil treatments (Treatments 3 and 5). In Soil Treatment 6, the EPI in cysts was higher (6.6) than in females (4.9). In the sterilized soil (Treatment 7), EPI in cysts was higher than in females, indicating the females and cysts used in this study were not originally free of fungi, and more eggs were originally colonized by fungi in cysts than in the females.

Fungal activities varied among the soils. The soil of Treatment 6, which has been in soybean monoculture for 29 years, showed the highest fungal parasitism of eggs. The soil of Treatment 4, which had been in corn/soybean annual rotation and was infested with a high density of *H. glycines,* also had high fungal parasitism of eggs (EPI 3.1 for females). Egg-parasitic index for *V. chlamydosporium* in soil (Treatment 2) was 3.0 (Table 2), substantially less than on agar (9.5). ARF18 colonized a low percentage of eggs both in females and cysts in soil (Treatment 3). The soil of Treatment 1 (sandy) and the

TABLE 2. Fungal parasitism of *Heterodera glycines* eggs in white to yellow females and in brown cysts in various soils—Test 3.

Soil treatment[b]	Egg-parasitic index (EPI)[a]	
	In females	In cysts
1. Sandy loam (Soil 1), without *H. glycines*	1.8 cA	0.9 cB
2. Soil 1 + *V. chlamydosporium*	3.0 bA	1.6bB
3. Soil 1 + ARF18	1.6cdA	1.4bA
4. Sandy loam, with high density of *H. glycines*	3.1 bA	1.5bB
5. Clay loam, without *H. glycines*	1.1 dA	0.9 cA
6. Clay loam, with *H. glycines,* soybean monoculture	4.9 aB	6.6aA
7. Sterilized Soil 1	0.4 eB	0.9 cA

Data are means of eight replicates. The same lowercase letters in columns or uppercase letters in rows indicate no significant difference at $P = 0.05$ according to LSD test.

[a] Egg-parasitic index: 0 = no eggs colonized, 1 = 1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, and 10 = 91–100% eggs colonized.

[b] Detailed descriptions of the soil treatments are provided in the text.

TABLE 1. Fungal parasitism of *Heterodera glycines* eggs in white to yellow females and in brown cysts on agar.

Test 1			Test 2		
	Egg-parasitic index (EPI)[a]			Egg-parasitic index (EPI)[a]	
Fungus	In females	In cysts	Fungus	In females	In cysts
Cylindrocarpon destructans isolate 1	3.4A	0.1B	ARF18	6.6A	2.9B
isolate 2	2.7A	0.5B	*Cylindrocarpon destructans* isolate 5	4.0A	2.1B
isolate 3	2.6A	0.6B	*Fusarium oxysporum* isolate 3	4.3A	1.3B
isolate 4	3.3A	0.5B	*Fusarium solani* isolate 2	1.7A	1.1B
Fusarium oxysporum isolate 1	3.2A	1.0B	*Pyrenochaeta terrestris*	2.2A	1.1B
isolate 2	4.5A	0.8B	*Verticillium chlamydosporium*		
Fusarium solani isolate 1	2.1A	0.7B	Florida isolate	9.4A	1.8B
Gliocladium catenulatum isolate 1	0.7A	0.1B	Sterile fungus 1	7.9A	4.5B
isolate 2	0.6A	0.1B	Control	0.1B	0.8A
Phialophora sp.	0.4A	0.3A			
Phoma sp.	4.2A	0.5B			
Verticillium chlamydosporium					
Minnesota isolate	2.4A	0.3B			
Florida isolate	9.5A	2.4B			
A-1-24	0.9A	0.1B			
Control	0 A	0.1A			

Data are means of three (Test 1) or five (Test 2) replicates. The different letters in rows within the same test indicate no significant difference at $P = 0.05$ according to LSD test.

[a] Egg-parasitic index: 0 = no eggs colonized, 1 = 1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, and 10 = 91–100% eggs colonized.

soil of Treatment 5 (clay), which were not infested with *H. glycines,* showed low fungal parasitism of eggs.

Effects of pre-colonization of cysts on egg parasitism by fungi: Tests 4 and 5. Pre-colonization of cysts by an unidentified isolate A-1-24 generally did not reduce colonization of eggs by most of the fungi tested on water agar (Table 3). However, the EPI of *C. destructans* isolate 3 was higher ($P = 0.004$) in non-pre-colonized cysts than in cysts pre-colonized by A-1-24. In contrast, the EPI of *F. solani* isolate 1 was higher ($P < 0.05$) in cysts pre-colonized by A-1-24 than in non-pre-colonized cysts (Table 3). When *C. cochliodes* was used as the pre-colonizing fungus, *V. chlamydosporium* Florida isolate, *F. oxysporum* isolate 3, *F. solani* isolate 2, ARF18, and Sterile Fungus 1 colonized fewer eggs in the pre-colonized cysts than in the cysts that were not pre-colonized (Table 3).

Test 6. No interactive effects of pre-colonization and soil treatment on EPI were observed. There was no significant difference in EPI between the cysts that were pre-colonized by *C. cochliodes* and the cysts that were not pre-colonized in soil infested with natural or introduced parasitic fungi (Table 4). However, egg parasitism differed among soils (Table 4).

Test 7. Pre-colonization of cysts by different fungi had different effects on subsequent colonization of the eggs in the cysts by the two highly pathogenic fungi, *V. chlamydosporium* and ARF18 (Table 5). Egg-parasitic index of *V. chlamydosporium* was not different between the cysts that were not pre-colonized and the cysts that were pre-colonized by *C. destructans, F. oxysporum,* and *F. solani.* However, pre-colonization of cysts by *Mortierella* sp., *P. terrestris,* and *C. cochliodes* suppressed the subsequent parasitism of eggs by *V. chlamydosporium*. Pre-colonization of cysts by fungi, except for *Mortierella* sp., reduced EPI in the cysts subsequently exposed to ARF18. Subsequent treatment with the *V. chlamydosporium* or ARF18 generally increased EPI in the cysts whether or not pre-colonized by a fungus as compared with the treatment without *V. chlamydosporium* or ARF18. However, when cysts were pre-colonized by *C. destructans,* subsequent treatment with ARF18 did not increase EPI. When cysts were pre-colonized by *F. oxysporum,* neither *V. chlamydosporium* nor ARF18 increased EPI.

Test 8. As in the agar test, different fungi had different effects on parasitism of the eggs in cysts by the subsequent colonizers, *V. chlamydosporium* and ARF18 in soil (Table 5). The EPI of *V. chlamydosporium* were not different between the cysts that were not pre-colonized and the cysts that were pre-colonized by *C. destructans* or *F. solani*. However, pre-colonization of cysts by *F. oxy-*

TABLE 4. Fungal parasitism of *Heterodera glycines* eggs in cysts in various soils as influenced by pre-colonization of the cysts by a non-parasitic fungus—Test 6.

Soil treatment[b]	Egg-parasitic index (EPI)[a]	
	Non-pre-colonized	Pre-colonized[c]
1. Sandy loam (Soil 1), without *H. glycines*	1.9cA	2.1bcA
2. Soil 1 + *V. chlamydosporium*	3.0bA	2.3bA
3. Soil 1 + ARF18	1.5cA	1.5bcA
4. Sandy loam, with high density of *H. glycines*	4.1aA	3.8aA
5. Clay loam, without *H. glycines*	1.8cA	1.5bcA
6. Clay loam, with *H. glycines,* soybean monoculture	4.1aA	3.7aA
7. Sterilized Soil 1	0.7dB	1.2cA

Data are means of eight replicates for each treatment. The same lowercase letters in columns or uppercase letters in rows indicate no significant difference at $P = 0.05$ according to LSD test.

[a] Egg-parasitic index: 0 = no eggs colonized, 1 = 1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, and 10 = 91–100% eggs colonized.

[b] Detailed descriptions of the soil treatments are provided in the text.

[c] The cysts were pre-colonized by an isolate of *Chaetomium cochliodes.*

TABLE 3. Fungal parasitism of *Heterodera glycines* eggs in cysts on agar as influenced by pre-colonization of the cysts by a non-parasitic fungus.

Test 4			Test 5		
	Egg-parasitic index (EPI)[a]			Egg-parasitic index (EPI)[a]	
Fungus	Non-pre-colonized	Pre-colonized[b]	Fungus	Non-pre-colonized	Pre-colonized[b]
Cylindrocarpon destructans isolate 1	3.6A	2.4A	ARF18	8.1A	5.0B
isolate 3	2.9A	1.5B	*Cylindrocarpon destructans* isolate 5	4.1A	3.2A
Fusarium oxysporum isolate 1	2.9A	2.7A	*Fusarium oxysporum* isolate 3	4.3A	2.0B
Fusarium solani isolate 1	2.1B	3.2A	*Fusarium solani* isolate 2	2.8A	2.1B
Gliocladium catenulatum isolate 2	0.5A	0.4A	*Pyrenochaeta terrestris*	3.0A	3.3A
Verticillium chlamydosporium Florida isolate	6.8A	6.4A	*Verticillium chlamydosporium* Florida isolate	7.4A	4.7B
Minnesota isolate	2.1A	1.5A	Sterile fungus 1	7.8A	4.4B
Control	0 B	1.0A	Control	0.1B	1.4A

Data are means of three (Test 4) or five (Test 5) replicates. The same letters in rows within the same test indicate no significant difference at $P = 0.05$ according to LSD test.

[a] Egg-parasitic index: 0 = no eggs colonized, 1 =1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, and 10 = 91–100% eggs colonized.

[b] In Test 4, the cysts were pre-colonized by an unidentified fungal isolate A-1-24; in Test 5, the cysts were pre-colonized by an isolate of *Chaetomium cochliodes.*

TABLE 5. Parasitism of *Heterodera glycines* eggs by the pathogenic fungi *Verticillium chlamydosporium* and ARF18 as influenced by pre-colonization of the cysts by individual fungi with various degrees of pathogenicity to eggs.

	Egg-parasitic index (EPI)[a]					
	Test 7 (on agar)			Test 8 (in soil)		
	Vc[b]	ARF18	Without Vc or ARF18	Vc[b]	ARF18	Without Vc or ARF18
Cylindrocarpon destructans isolate 5	7.0aA	5.4bB	4.4aB	4.1aA	3.2aA	3.7aA
Fusarium oxysporum isolate 3	5.7abA	5.2bA	4.9aA	2.2bcA	2.3bA	1.6cdA
Mortierella sp.	5.0bB	6.9aA	1.3cC	1.6cB	2.3bA	1.9cB
Pyrenochaeta terrestris	4.3bA	3.6cA	2.3bB	1.6cB	2.0bcB	2.6bA
Fusarium solani isolate 2	6.8aA	5.2bA	3.1bB	3.0abA	1.5cB	0.7eC
Chaetomium cochliodes	4.7bA	5.0bA	1.4cB	2.3bcA	1.8bcAB	1.2dC
Control (no pre-colonizing fungus)	7.4aA	8.1aA	0.1dB	3.7aA	1.4cB	1.2dB

Data are means of five (Test 7) or eight (Test 8) replicates. The same lowercase letters in columns or uppercase letters in rows within the same test indicate no significant difference at $P = 0.05$ according to LSD test.

[a] Egg-parasitic index: 0 = no eggs colonized, 1 = 1–10%, 2 =11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, and 10 = 91–100% eggs colonized.

[b] Vc = *Verticillium chlamydosporium.*

sporum, Mortierella sp., *P. terrestris,* or *C. cochliodes* suppressed parasitism of eggs by *V. chlamydosporium.* ARF18 showed little activity in the soil. In the cysts pre-colonized by *C. destructans, F. oxysporum,* and the cysts without pre-colonizer, there was no difference in EPI between the soil treated with ARF18 and soil without addition of the fungus. In the cysts pre-colonized by *Mortierella* sp., *F. solani,* and *C. cochliodes,* subsequent incubation in the soil treated with ARF18 appeared to increase EPI in the cysts as compared with the soil without addition of ARF18. The EPI in cysts pre-colonized by *P. terrestris* was lower in soil treated with *V. chlamydosporium* or ARF18 than in soil without addition of any fungus.

DISCUSSION

We showed that eggs with an embryo were more vulnerable to fungal attack than eggs containing J1 or J2. The reason behind this phenomenon, however, is still unclear. To colonize the embryo or juvenile, a fungus must penetrate the eggshell as well as the membrane of the embryo or the cuticle of the juvenile. Because the structure and chemical components of the membrane of an embryo and the cuticle of a juvenile are dramatically different, it is possible that the juvenile cuticle is resistant to fungal parasitism. If this is true, it would be interesting to determine whether fungal penetration of eggshell affects the juvenile within the eggs even if the fungus doesn't parasitize the juvenile.

Soil mycoflora is complex, with numerous species belonging to various taxonomic groups (Domsch et al., 1980). Although a number of fungi have been isolated from nematode cysts, they represent only a few taxonomic groups as compared with the total number of fungal species present in agricultural soils (Morgan-Jones and Rodríguez-Kábana, 1988; Rodríguez-Kábana and Morgan-Jones, 1988), indicating the niche represented by the cysts may be suitable for certain fungi that have adapted to it. Our studies demonstrated that pre-colonization of a cyst by a fungus generally reduced parasitism of eggs in the cysts by a subsequent parasitic fungus. Whether the pre-colonizer prevented other fungi from entering the cysts or inhibited infection of eggs after they entered the cysts is unclear. Presumably, reduction of fungal parasitism of eggs by the pre-colonizing fungus was due to inhibitory effect on growth of the parasitic fungi. Competition among fungi or fungistasis has been demonstrated (Mankau, 1962). Presence of some fungi may affect other fungi through nutritional depletion, space occupation, and toxic activities.

The interaction between the pre-colonizing fungi and the parasitic fungi depended on the combination of the fungal species or isolates. In some cases, pre-colonization of cysts had no effect on parasitism of eggs by other fungi. It appeared that pre-colonization of cysts by the unidentified fungus isolate A-1-24 had little effect on subsequent parasitism of eggs by other fungi on agar (Table 3). In contrast, pre-colonization of cysts by *C. cochliodes* generally suppressed parasitism of eggs by other fungi, except for *C. destructans* isolate 5 and *P. terrestris* (Table 3). *Chaetomium cochliodes* has been isolated from females and cysts of *H. glycines* (Carris et al., 1989; Chen and Chen, 2002; Chen et al., 1994), and colonization of cysts by the fungus may protect the eggs from infection by other fungi. Pre-colonization of cysts on agar by *C. destructans, F. oxysporum,* and *F. solani*—three fungi with moderate pathogenicity (Chen et al., 1996b)—did not significantly reduce EPI of *V. chlamydosporium.* However, it could not be determined whether the pre-colonizers had no effect on parasitism of eggs by *V. chlamydosporium* or the sum of the parasitism by the two fungi, *C. destructans/F. oxysporum* and *V. chlamydosporium,* was equal to the parasitism of eggs by *V. chlamydosporium* alone. On agar, ARF18 was suppressed by all fungi except the *Mortierella* sp., which appeared to

be weakly pathogenic to eggs. Egg-parasitic index was higher in cysts pre-colonized by *Mortierella* sp. than without pre-colonization both on agar and in soil containing ARF18. Further study is needed to determine whether there is synergistic interaction in growth or in parasitism of eggs by the two fungi.

Environmental conditions may be important in determining the outcome of pre-colonization of cysts on parasitism of eggs by other fungi. The results from agar tests may not be extrapolated directly to the situation in soil. There was no interaction between pre-colonizing fungus and medium (agar vs. soil) on EPI in cysts subsequently treated with *V. chlamydosporium* (Table 5, data analysis not shown), indicating that the trends of pre-colonization of cysts by various fungi on subsequent parasitism of eggs by *V. chlamydosporium* were similar on agar and in soil. However, significant interaction between pre-colonizing fungus and medium on EPI was observed in cysts subsequently treated with ARF18. On agar, ARF18 had a high EPI. In soil, the fungus had limited egg-parasitism, probably due to slow growth, leading to poor establishment of ARF18 by the time the experiment was terminated.

For successful biological control of cyst nematodes with an egg-parasitic fungus, it is crucial that the fungus be highly pathogenic to the eggs, and able to compete with soil fungi and colonize females in which the eggs are in early developmental stages. The fungi most commonly isolated from females and cysts of *H. glycines* were *Fusarium* spp., *Cylindrocarpon* spp., *Paecilomyces* spp., *Exophiala* spp., *Gliocladium* spp., *Phoma* spp., *Stagonospora heteroderae*, and *Neocosmospora vasinfecta* (Bernard et al., 1997; Carris et al., 1989; Chen and Chen, 2002; Chen et al., 1994; Gintis et al., 1983; Morgan-Jones et al., 1981). Most of these fungi are fast growing. If they are able to enter the females through cuticle or natural openings, they may have a better chance to colonize females containing eggs in early developmental stages than slow-growing fungi. However, they are only moderately pathogenic to *H. glycines* eggs. Consequently, colonization of cysts by these fungi may prevent other highly pathogenic fungi from colonizing the cysts. Highly pathogenic fungi, such as the black, yeast-like fungus (Chen et al., 1996b) and the sterile fungus used in this study, are slow growing or extremely slow growing on mycological media. Although ARF18 and *V. chlamydosporium* grow faster on these media, they may not be able to compete with other fast-growing fungi such as *Fusarium* in soil and cysts. Timper and Riggs (1998) demonstrated that ARF-L isolates, which are less pathogenic to eggs on agar, were more effective in suppressing *H. glycines* densities in soil than ARF-C isolates. One of the reasons could be that the ARF-L isolates are more competent in the rhizosphere and are able to rapidly colonize females containing eggs at early developmental stage when they are susceptible to parasitism. Efforts are still needed to search for fast-growing, highly pathogenic fungi or develop such fungi with genetic engineering for effective biological control of cyst nematodes.

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