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Variation in Efficacy of Isolates of the Fungus ARF Against the Soybean Cyst Nematode *Heterodera glycines*

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Abstract: An unnamed fungus, designated ARF, that parasitizes eggs and sedentary stages of cyst nematodes is a potential biological control agent of *Heterodera glycines*. The objectives of this study were to determine whether ARF isolates differ in their ability to suppress nematode numbers in soil and to compare the efficacy of ARF in heat-treated and native soil. The effectiveness of 11 ARF isolates was compared by introducing homogenized mycelium into heat-treated soil. Soybean seedlings were transplanted into pots containing fungus-infested soil and inoculated with *H. glycines*. After 30 or 60 days, the number of nematodes and the percentage of parasitized eggs were determined. Three isolates (907, 908, and TN14), which were previously reported to be weak egg parasites in vitro, consistently suppressed nematode numbers by 50% to 100%. Of the isolates previously reported to be aggressive egg parasites, four (903, BG2, MS3, and TN12) reduced nematode numbers by 56% to 69% in at least one experimental trial, but the other four had no effect on nematode numbers. When the efficacy of isolate TN14 was tested in heat-treated and native soil, nematode suppression was greater in the heat-treated soil in only one of two trials. In both soil treatments, nematode numbers were reduced by more than 60%. We conclude that virulence toward nematode eggs in vitro is a poor indicator of effectiveness of an ARF isolate in soil, and that the presence of soil microbes may reduce, but does not completely inhibit, activity of isolate TN14.

Key words: ARF, Arkansas Fungus 18, biological control, efficacy, *Heterodera glycines*, nematode, nematophagous fungus, soybean, soybean cyst nematode, suppression.

The soybean cyst nematode *Heterodera glycines* Ichinohe is managed by rotating susceptible soybean (*Glycine max*) cultivars with resistant cultivars and nonhost crops (Young, 1992a). However, in many fields resistance cannot be sustained due to population shifts in race and species of nematode (Young, 1992b). Moreover, in the northern regions of the United States, eggs within cysts remain viable in the soil for several years, necessitating longer rotations with nonhost crops (Niblack, 1993). Biological control, if successful, could be integrated with rotation to slow the development of virulent populations and to shorten the rotation out of soybean.

The fungus ARF (Arkansas Fungus 18) is a promising biological control agent of cyst nematodes. In greenhouse experiments the BG2 isolate suppressed *H. glycines* populations by 86% to 99% on nine soybean cultivars (Kim and Riggs, 1995). The fungus infects all sedentary stages of *H. glycines*, including eggs, third- and fourth-stage juveniles, and females (unpubl. data). ARF is characterized by its ability to infect *H. glycines* eggs, produce sterile white mycelium, and form sclerotium-like structures on cornmeal agar (Kim and Riggs, 1991). The lack of sexual or asexual spores has prevented classification of this fungus, but the presence in the hyphae of a simple septum with a central septal pore and nearby Woronin bodies strongly indicates that ARF is in the Ascomycotina (Kim et al., 1992).

Several isolates exhibiting characteristics of ARF have been collected, most originating from the mid-south region of the United States. Based on morphology of the sclerotium-like structures, isolates were placed into two groups (Kim et al., 1998), designated compact (ARF-C) and loose (ARF-L). Analysis of mitochondrial DNA restriction fragment length polymorphisms (mtDNA RFLP) using three restriction enzymes and two mtDNA probes showed that the isolates belong to four different haplotypes (Kim et al., 1998). The ARF-C isolates were all in haplotype I. The ARF-L isolates were classified into haplotypes II, III, and IV. Whether these mtDNA RFLP haplotypes represent

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different species or different biotypes of the same species is unclear.

In an in vitro assay, isolates of ARF differed in their virulence as egg parasites. When cysts were placed on mycelia growing on agar, the percentage of eggs parasitized after 10 days ranged from 64% to 93% for the ARF-C isolates and 22% to 40% for the ARF-L isolates (Kim et al., 1998). We hypothesized that the ARF-C isolates, because they were aggressive egg parasites, would be more effective than the ARF-L isolates in suppressing nematodes in the rhizosphere of soybean. Therefore, our first objective was to determine whether ARF isolates differ in their ability to suppress nematode numbers in heat-treated soil. Because heat-treating soil may reduce antagonists (fungi and bacteria) of ARF that in native soil may inhibit the growth or survival of the fungus, our second objective was to compare the efficacy of ARF in heat-treated and native (unheated) soil.

Materials and Methods

Heterodera glycines race 3 was cultured on soybean cv. Lee 74 in a greenhouse where temperatures ranged from 27 to 35 °C. Cysts were extracted by hand-rubbing roots in water, collecting them on a 250-µm-pore sieve nested under an 850-µm-pore sieve, and then removing root and soil debris using centrifugal flotation (Southey, 1986). Cysts were crushed with a tissue grinder to release the eggs, and the eggs were placed in hatching chambers to obtain second-stage juveniles (J2). Hatching chambers were constructed by placing a slightly concave wire screen inside a 15-cm petri dish containing ca. 80 ml of 16 mM $ZnSO_4 \cdot 7H_2O$ to increase the rate of egg hatch (Tefft and Bone, 1984). Three layers of circular-cut tissue paper were laid over the screen and eggs poured onto the tissue. Second-stage juveniles were collected every 3 to 4 days by pouring the $ZnSO_4$ over a 25-µm-pore sieve and rinsing with tap water. Fresh $ZnSO_4$ was added to the chambers after each collection.

The ARF isolates originated from infected *H. glycines* eggs collected in the mid-south region of the United States from 1987 to 1994 (Table 1). The isolates have been continuously cultured at room temperature (20–26 °C) on agar medium. Inoculum of ARF was prepared by grinding two 1-cm-diam. plugs from the leading edge of a colony growing on Emerson's YpSs medium (1.5% agar) in a sterile microfuge tube containing 0.5 ml water, and pouring the mycelial fragments into 150 ml of sterile pea juice medium (2:3 v:v canned pea juice:distilled water) in a 250-ml flask. The flasks were placed on a rotary shaker at room temperature (20–26 °C) for 7 to 9 days. Mycelium was harvested by vacuum-filtering the liquid medium through a Whatman No. 3 filter and then rinsing three times with distilled water. Excess water was removed by pressing the mycelium between several layers of paper towel. A known wet weight of mycelium (1 g wet weight is equivalent to ca. 0.2 g dry weight) was added to 30 to 50 ml of water and homogenized in a Waring blender at medium speed for three 10-second intervals. The homogenized mycelium was added to a measured volume of dry soil and thoroughly incorporated by hand. Unless otherwise noted, the soil was a silt (8% sand, 84% silt, 8% clay; pH 4.0) that had been steam-heated to 105 °C for 3 hours to kill most soil organisms.

Table 1. Sources of ARF isolates used in this study.

Isolate	SLS[a]	Location[b]	Year of isolation	ARSEF No.[c]
903	C	Woodruff Co., AR	1989	5238
907	L	Pope Co., AR	1989	5240
908	L	Pope Co., AR	1989	5241
BG2	C	Washington Co., AR	1987	5239
KY1	C	McCracken Co., KY	1994	5244
MS1	C	Desoto Co., MS	1994	5245
MS3	C	Desoto Co., MS	1994	5246
MS5	C	Tate Co., MS	1994	5247
MS8	C	Grenada Co., MS	1994	5248
TN12	C	Lauderdale Co., TN	1994	5242
TN14	L	Lauderdale Co., TN	1994	5243

[a] Morphology of sclerotium-like structures (SLS): C = compact, L = loose.

[b] County and state in which isolates were obtained from infected *Heterodera glycines* eggs. Isolates from the same county were from different fields or different locations in the same field.

[c] Isolate accession numbers in the ARS Collection of Entomopathogenic Fungi (ARSEF), Ithaca, NY.

Two experiments were used to determine whether the ARF isolates differed in their ability to suppress *H. glycines* numbers on susceptible plants. In the first experiment, soil containing 0.002 g mycelium/cm^3 was packed into 50-cm^3 conical plastic pots (16.5 × 2.7 cm, length × upper diam.) and a single 7- to 9-day-old Lee 74 soybean seedling was transplanted into each pot. The seedling roots were trimmed prior to planting so that the root system could fit into a 3 × 1-cm hole. After 1 week of plant growth, 222 ± 14 ($\bar{x}$ ± SE) J2 and 137 ± 20 eggs were added in water to the soil surface. The treatments were randomly arranged in a greenhouse maintained at 27 to 35 °C. Thirty days after adding nematodes to the pots, cysts and J2 were extracted from the roots and soil using wet-sieving followed by centrifugal flotation (Southey, 1986). Cysts were crushed with a tissue grinder to release the eggs, which were then collected on a 25-µm-pore sieve. The eggs and J2 from the cysts, and the J2 from the soil, were counted. To determine the proportion of eggs parasitized by a fungal isolate, eggs from each pot were concentrated in a conical centrifuge tube and the water replaced with 10% formaldehyde. Drops of concentrated eggs were covered with glass slips, and the first 30 eggs encountered were examined at ×500 for the presence of internal mycelium. In cases where there were fewer than 50 eggs/pot, the proportion parasitized was not determined because of the difficulty in finding eggs. The percentage of parasitized eggs per pot was used to determine the number of unparasitized eggs. There were five to eight replicate pots per ARF isolate and control (soil without fungus). The efficacy of all isolates was tested in at least two trials. Differences among isolates were determined with analysis of variance (SAS Institute, Cary, NC) on unparasitized eggs transformed by square root. Fisher's LSD test was used to separate means.

Due to concerns that the eggs produced by the nematodes in the first experiment were exposed to the fungus for only a short time (≤15 days), a second experiment was conducted in which eggs were exposed to the fungus for a longer period of time. In this experiment, soil containing 0.003 g mycelium/cm^3 soil was added to 500-cm^3 clay pots (4-cm-diam.), and two Lee 74 soybean seedlings with trimmed roots were transplanted into the soil of each pot. After 1 week of plant growth, a total of 4,176 ± 180 *H. glycines* eggs were pipetted into four 2-cm-deep holes near the plant stems (two holes per plant). The holes were covered with soil, and the pots were randomly arranged in a greenhouse maintained at 27 to 35 °C. Cysts and J2 were extracted from roots and soil after 60 days, cysts were crushed as described in the previous experiment, and the number of eggs and J2 was counted. In the first two of three experimental trials, the percentage of eggs with internal mycelium was determined. There were five to seven pots per isolate and control (soil without fungus). Isolates 908, BG2, MS3, KY1, TN12, and TN14 were tested in at least two trials; isolates 903 and 907 were tested in only one trial. The statistical analysis was the same as described in the previous experiment.

To compare the efficacy of ARF in heat-treated and native (nonheated) soil, a very fine sandy loam (57% sand, 27% silt, 16% clay; pH 6.2) with no detectable population of *H. glycines* was used. The soil was collected from a field on the University of Arkansas Experiment Station, Fayetteville, in August and October 1996. The field was planted to pumpkin during the first soil collection and was follow during the second collection. Within a week of collection, a portion of the soil was moistened, placed in 500-cm^3 clay pots, and heated in a microwave oven set at full power for 1.5 minutes. Soil temperature reached a maximum of 80 to 90 °C during heating. Microwave heating of soil reduces the total viable fungal biomass, and to a lesser extent prokaryote biomass (Ferriss, 1984). Heat-treated and native soil was air-dried before mycelium of isolate TN14 was mixed into it at concentrations of 0.0005 and 0.001 g/cm^3 soil. Water without mycelium was mixed into heat-treated and native soil as controls. The soil was added to 50-cm^3 conical pots and planted with 7- to 9-day-old Lee 74 soybean seedlings. After 1 week of

plant growth, 202 ± 14 J2 and 110 ± 2 eggs were pipetted onto the soil surface. Cysts and J2 were extracted from the roots and soil 30 days later, the cysts were crushed, and the number of eggs and juveniles was counted. Egg parasitism was not determined. The relationship between fungal concentration and $\log_{10}$ (nematode numbers) in heat-treated and native soil was determined with regression analysis (SAS Institute, Cary, NC). Differences in the slopes and intercepts between heat-treated and native soil was determined with *t* tests.

Results

The ARF isolates differed in their ability to reduce numbers of *H. glycines* after 30 days ($P \leq 0.05$). The ARF-L isolates (907, 908, and TN14) suppressed nematode numbers by 50% to 99% compared to the control, whereas many of the ARF-C isolates had no effect or were only moderately suppressive (Fig. 1). The most effective ARF-C isolate was TN12. In Trials 1 and 3, this isolate reduced nematode numbers by 35% (not significantly different from the control) and 69%, respectively. Few eggs (<50/pot) were present in pots containing the ARF-L isolates in Trials 2, 3, and 4. Consequently, egg parasitism was not determined for these isolates. Egg parasitism averaged less than 5% for most of the isolates except 907, MS8, TN12, and TN14 (Table 2). Less than 1% of the eggs were parasitized in the control pots.

The results of the 60-day experiment were similar to the 30-day experiment in that numbers of *H. glycines* were lower ($P \leq 0.05$) in pots containing the ARF-L isolates than in pots containing the ARF-C isolates (Fig. 2). The three ARF-L isolates suppressed nematode numbers by 80% to 100%, and the ARF-C isolates 903, BG2, and MS3 suppressed numbers by 56% to 65% compared to the control. The other ARF-C isolates did not suppress nematode numbers. Egg parasitism was generally higher in the 60-day compared to the 30-day experiment (Table 2).

In both trials and soil treatments (heat-treated and native), nematode numbers decreased with increasing concentration of isolate TN14 (Fig. 3). In trial 1, the rate of nematode decline (slope) was greater in heat-treated than in native soil ($P \leq 0.0001$), whereas, in trial 2, the decline was greater in native than in heat-treated soil ($P \leq 0.001$). There was no difference between native and heat-treated soil in number of nematodes in pots without fungus (intercept).

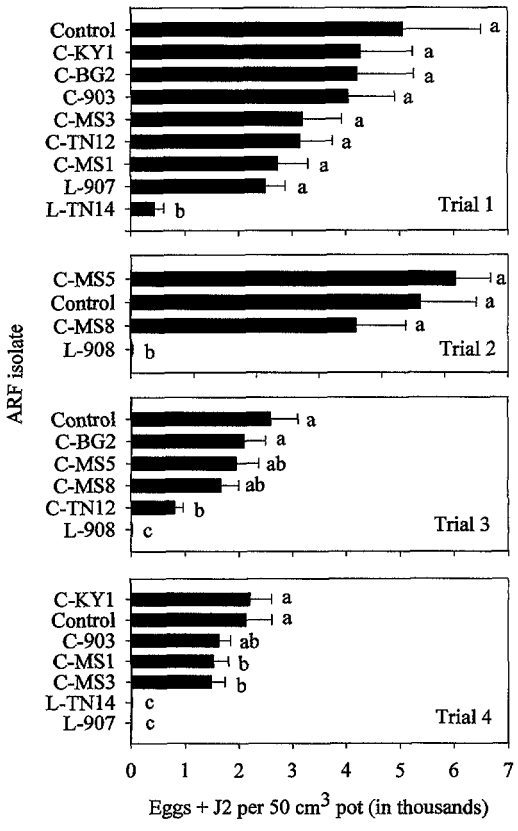

FIG. 1. Effect of ARF isolates on the number of unparasitized eggs and second-stage juveniles (J2) of *Heterodera glycines* 30 days after addition of nematodes. Heat-treated soil was infested with ARF by mixing homogenized mycelium at a rate of 0.002 g/cm^3 soil (except the control). Bars are the means ± SE of five to eight replicate pots. Differences ($P \leq 0.05$) among means are denoted by letters above the error bars. The letters preceding the isolate designations identify isolates forming compact (C) and loose (L) sclerotium-like structures on cornmeal agar.

Discussion

In experiments on agar, the ARF-C isolates were more aggressive parasites of *H. glycines* eggs than were the ARF-L isolates

TABLE 2. Percentage of eggs parasitized by ARF isolates in the 30- and 60-day experiments.

ARF isolate	Percentage of parasitized eggs[a]	
	30-day experiment	60-day experiment
Control	0.6 ± 0.3 (26)	0.2 ± 0.2 (12)
C-903[b]	1.9 ± 1.2 (13)	24.7 ± 6.2 (7)
L-907	11.2 ± 5.6 (6)	—
L-908	—	17.6 ± 9.1 (5)
C-BG2	2.1 ± 0.9 (9)	24.8 ± 7.3 (12)
C-KY1	0.7 ± 0.5 (13)	11.6 ± 7.4 (5)
C-MS1	1.2 ± 1.0 (12)	—
C-MS3	0.7 ± 0.5 (13)	17.4 ± 5.6 (5)
C-MS5	4.5 ± 3.8 (14)	—
C-MS8	8.8 ± 3.9 (15)	—
C-TN12	15.6 ± 6.0 (9)	2.8 ± 1.2 (5)
L-TN14	29.6 ± 6.0 (6)	43.7 ± 7.4 (7)

[a] Mean percentage ± SE of parasitized eggs after 30 or 60 days. Means are the combined data from one to four experimental trials. Egg parasitism was determined by examining 30 eggs at random for the presence of internal mycelium. Numbers in parentheses represent the number of pots in which egg parasitism was determined. If fewer than 50 eggs/pot were recovered, then egg parasitism was not determined.

[b] The letters preceding the isolate designations identify isolates forming compact (C) and loose (L) sclerotium-like structures on cornmeal agar.

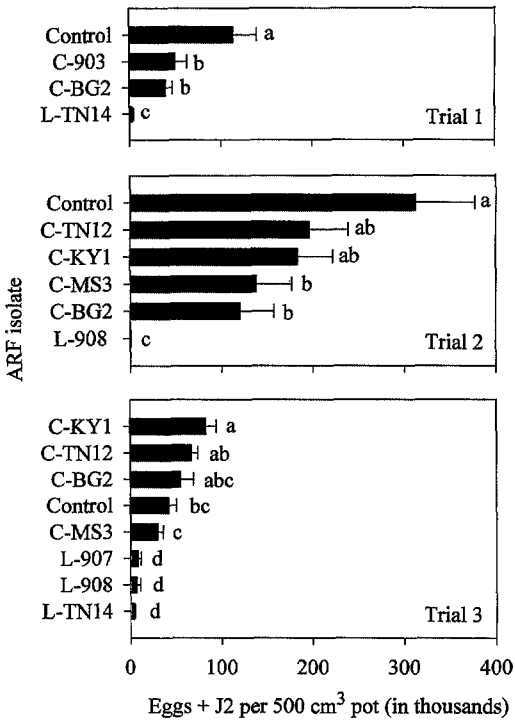

FIG. 2. Effect of ARF isolates on the number of eggs and second-stage juveniles (J2) of *Heterodera glycines* 60 days after addition of nematodes. Data for trials 1 and 2 include unparasitized eggs, while trial 3 includes total eggs (parasitized and unparasitized). Heat-treated soil was infested with ARF by mixing homogenized mycelium at a rate of 0.003 g/cm^3 soil (except the control). Bars are the means ± SE of five to seven replicate pots. Differences ($P \leq 0.05$) among isolates are denoted by letters above the error bars. The letters preceding the isolate designations identify isolates forming compact (C) and loose (L) sclerotium-like structures on cornmeal agar.

(Kim et al., 1998). However, in our 30- and 60-day soil experiments, the ARF-L isolates were more effective in suppressing *H. glycines* numbers than were the ARF-C isolates. The ARF-C isolates apparently had not lost their pathogenicity during prolonged subculture because trends in egg parasitism similar to those reported by Kim et al. (1998) were obtained (unpubl. data) when we repeated their in vitro assay. The discrepancy between the ability of ARF isolates to parasitize eggs on agar and to suppress nematode numbers in soil may be due to differences among the isolates in their specialization for different nematode stages or their activity and survival in soil.

Few eggs were found in pots containing the ARF-L isolates, suggesting that either the eggs were completely consumed by hyphae before the end of the experiment or the nematodes were killed before eggs were produced. It is possible that ARF-L isolates are aggressive parasites of sedentary juveniles and females while ARF-C isolates are aggressive parasites of eggs. Isolates that are able to penetrate the chitin/protein complex of the nematode egg shell (Wharton, 1986) may possess a different complement of enzymes than do those that are able to penetrate the collagen-like protein of the nematode cuticle. A concern in the first experiment was that the design was more conducive to parasites of juveniles and adults than to parasites of eggs due to the length of time the different stages were available for parasitism. In the first experiment, eggs and J2 were inoculated and the cysts extracted 30 days later. At an average greenhouse temperature of 31 °C, egg production would begin 15 days after initial penetration of roots by J2 (Ichinohe, 1959) and continue up to the time of extraction from the roots. Therefore, exposure of eggs to the fungus would be ≤15

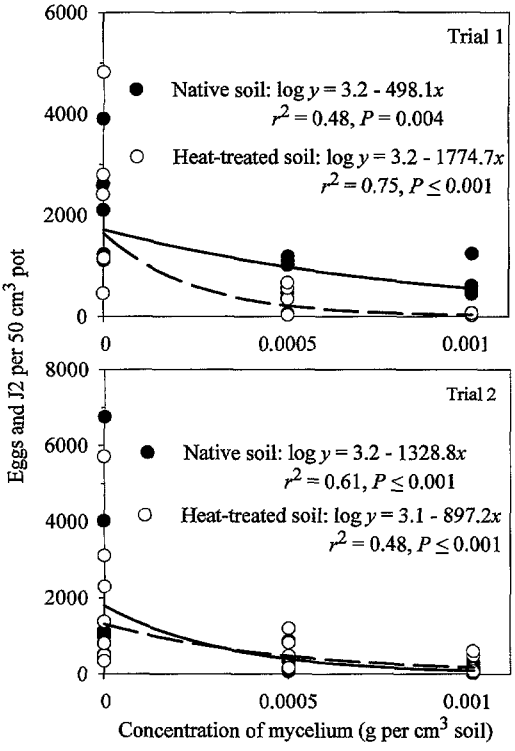

FIG. 3. Relationship between concentration of ARF isolate TN14 and number of *Heterodera glycines* in heat-treated and native soil 30 days after addition of nematodes. The fungus was incorporated into soil as homogenized mycelium. Data points represent the total number of eggs (parasitized and unparasitized) and second-stage juveniles per pot. There were five to seven replicate pots per fungus concentration and control.

days. In the second experiment, cysts were extracted 60 days after application of nematode inoculum to increase the exposure time of eggs. Nevertheless, even in the longer experiment, the ARF-L isolates more effectively suppressed nematode numbers than did the ARF-C isolates.

Differences in efficacy between the ARF-C and -L isolates may also be due to their ability to grow in bulk or rhizosphere soil. Fungal parasites of nematodes that can grow beyond the point of application, particularly in the rhizosphere, will have a greater probability of contacting nematode hosts than those that cannot grow, all other traits being equal. The ARF-C isolates may be aggressive parasites of nematode eggs but have a low capacity for growth in soil. De Leij and Kerry (1991) showed that several isolates of the fungus *Verticillium chlamydosporium* parasitized nematode eggs in vitro; however, only one was capable of growing in the rhizosphere, and it was the only isolate effective in nonsterile soil. The TN14 isolate of ARF suppressed nematode numbers in native soil even at a low infestation level (0.0005 g mycelium/cm^3 soil). Although this does not prove that the TN14 isolate can grow saprophytically in bulk or rhizosphere soil, the results indicate that activity of the isolate was not appreciably inhibited by the microbial community. Reducing the initial microbial community by heating the soil had no consistent effects on TN14; efficacy of the isolate was improved in one of two trials. The reasons for this inconsistency are unclear. The soil used in the two trials was collected from the same location but at different times (August and October 1996). Perhaps the soil community changed over time from one that was more inhibitory to one that was less inhibitory to TN14.

Caution must be used when employing in vitro screening for selecting potential biological control agents. Fungi that show poor efficacy in vitro may be effective under more natural conditions. For example, based on the results of Kim et al. (1998), one or more of the ARF-C isolates would have been selected as the most promising for further study because they were more virulent egg parasites than the ARF-L isolates. However, the ARF-L isolates were more effective in suppressing nematode populations in soil and are therefore better candidates for biological control of *H. glycines*. Clearly, traits in addition to virulence toward nematode eggs are involved in the efficacy of ARF, and these need to be determined.

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