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Efficacy of the Nematophagous Fungus ARF18 in Alginate-clay Pellet Formulations Against *Heterodera glycines*[1]

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Abstract: Dry alginate-clay pellets containing mycelium of ARF18 were added to sandy soil in greenhouse tests to determine the formulation's efficacy in the suppression of *Heterodera glycines*. Pellet formulation variables included quantity of mycelium per pellet (0.0–3.9%), pellet size (2.3 or 8.3 mg), pellet application rate per unit soil (0.4 or 1.0% based on dry soil weight), and pellet storage (0 or 90 days). All of these variables affected efficacy. Nematode suppression was greatest (95%) with 8.3 mg pellets containing 3.9% mycelium that were not stored and applied at the rate of 1.0% of dry soil weight. Storage for 90 days reduced the efficacy of the pellets. The soybean cultivars tested were not equally good hosts of *H. glycines*, but reproduction of the nematode was reduced equally on all. The average suppression was 96% (range 86–99%). Similar suppression of reproduction occurred in tests with six races of *H. glycines*. ARF18 appeared to be nonspecific with regard to soybean cultivar and *H. glycines* race.

Key words: biological control, carriers, egg parasite, formulation, fungus, *Glycine max*, *Heterodera glycines*, kaolin, nematode, nematophagus, race, soybean cyst nematode.

The nematophagous fungus ARF18 is an unidentified ascomycete (3). The fungus was isolated from a soybean field located in St. Francis County, Arkansas, in which the population density of *Heterodera glycines* had declined from 140 eggs/g soil in November 1986 to 4 eggs/g soil in July 1987 despite the planting of a susceptible soybean cultivar. ARF18 parasitizes eggs, second-stage juveniles (J2), and immature and mature females (3). It can penetrate directly through the cyst wall and parasitize eggs within the cyst (5). It grows well on several artificial media (4), but efforts to induce sporulation have been unsuccessful. A detailed morphological description is available (4,5).

Previous studies in a greenhouse demonstrated that incorporation of ARF18 into soil reduces population densities of *H. glycines* (3). Most of the previous research was conducted on one soybean cultivar and a few races of *H. glycines*. Other studies indicated that ARF18 did not survive well in storage in alginate formulations (3). Under refrigeration, it survived for only 6 months in alginate pellets. The objectives of our research were to evaluate the efficacy of various alginate-clay-mycelium formulations of ARF18 against *H. glycines*, and to determine whether soybean cultivar and nematode race influenced the efficacy of ARF18 in soil in a greenhouse. These studies were conducted in sterile soil with the understanding that ARF18 may not be as effective in non-sterile soil. In fact, it has been used in studies in non-sterile soil and has reduced reproduction 70–85% (Kim and Riggs, unpub.). Sterile soil was used to demonstrate the maximum effect; studies in non-sterile soil were delayed by changes in the status of the first author.

MATERIALS AND METHODS

Isolates and culture conditions: ARF18 was isolated from eggs of *H. glycines* that had been extracted from soil taken from a field on the Pine Tree Experiment Station, Colt, Arkansas (3). It was maintained on 1.5% cornmeal agar. Isolate BG2 was used for all studies.

The fungus was cultured on a modified liquid medium (11). A mycelium suspension (10^6 colony-forming units (cfu)/ml)

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was prepared from 10-day-old cultures of ARF18 grown in a 1:3 canned sweat pea juice:water medium. One-ml aliquants of the suspension were added to sterile 500-ml flasks containing 250 ml pea juice medium. The cultures were incubated on a gyrotary shaker at 100 rpm for 10 days at 21–24 C.

In an alternate culture method, 250 ml of a mycelial suspension (10^6 cfu/ml) prepared from 10-day-old cultures of ARF18 grown on pea juice medium was added to a sterile 15-liter fermentor (Microferm Fermentor, New Brunswick Scientific Co., New Brunswick, NJ) containing 12 liters of pea juice medium. The cultures were incubated for 10 days at 21–24 C with the bladed rotor running at 300 rpm continuously. An antifoam agent and sterile air (4 liters/minute) were supplied during fermentation. The spent medium containing mycelium of ARF18 was used in alginate-clay pellet formulations. To measure the yield of mycelium, the fungal cultures were filtered (Whatman 541, 11-cm-d filter, Whatman International Ltd., Maidstone, England) and the mycelial mat was dried at 45 C for 5 hours and weighed.

Mycelia from the two culture methods were mixed to prepare pellets for experimental work. The number of cfu per ml was determined from a dilution of one ml chopped mycelium in 10 ml water plated in 1-ml portions on potato dextrose agar in petri dishes. After 48 hours, the number of colonies that grew was recorded.

Formulation of alginate-clay pellets: The formulation process was conducted in a sterile hood, and all equipment used was previously cleaned with 1% NaOCl solution and washed with sterilized water. Alginate-clay pellets containing mycelia of ARF18 were prepared by the method of Walker and Connick (14) modified as follows. Spent medium containing mycelium (250, 500, or 750 ml) was blended in a blender for 30 sec at medium speed. While the blade was still running, sterile water (640, 390, or 140 ml, respectively), 100 g kaolin clay (Fluka Chemika, Ronkonkoma, NY), and 10 g alginate (Kelgin MV, Kelco, Chicago, IL) were added in order for a total volume of 1 liter. The alginate-mycelium-clay mixture was blended for 1 minute at maximum speed.

The alginate-clay-mycelium mixture was poured into a 1.9-liter-capacity garden sprayer (Model 050P, Gilmour, Somerset, PA), pressurized, and pumped through a rubber hose (0.6-mm inside diameter) into a funnel that was fitted with a plastic cap (12.5-cm-d), inverted, and held by a clamp on a support. The plastic cap had 47 equally spaced 2.2-mm-d holes. The mixture was forced through the holes, resulting in the formation of drops that fell into the 0.25 M $CaCl_2$ gellant solution to form 3–4-mm-d spherical beads. The beads were separated from the solution in <10 minutes and dried 2 days on a plastic screen (1-mm^2 openings) with a fan blowing across it and with occasional mixing.

Half of the dried pellets were ground in a Model 4E Quaker City Mill (Philadelphia, PA) to produce small pellets. Ground pellets were screened through nested 1.7-mm-aperture and 0.8-mm-aperture sieves, and pellets on the 0.8-mm-aperture sieve were saved. The large unground pellets weighed approximately 8.3 mg each, and the small pellets weighed approximately 2.3 mg each. All pellets were stored at 5 C in plastic bottles with screw caps.

The culture medium contained mycelium but no conidia or chlamydospores. One liter of spent medium yielded approximately 6.0 g dry (55 g wet) mycelium. When dried pellets were made with 250 ml spent medium, each liter of alginate-clay-mycelium mixture contained 1.5 g dry mycelium (1.3%), 10 g alginate (9.0%), and 100 g kaolin clay (89.7%). Thus, pellets made with 250 ml, 500 ml, and 750 ml of spent medium were approximately equivalent to 1.3%, 2.6%, and 3.9% dry mycelium per pellet, respectively. The dried pellet had 8.7% moisture.

Nematode sources: Isolates of six races of *H. glycines* were obtained from stock cultures maintained in a greenhouse on appropriate soybean cultivars. Races 2, 4, 9, and 14 were maintained on 'Pickett' and

Races 3 and 12 were maintained on 'Lee 74'.

Effect of pellet formulations on H. glycines: The efficacy of pellet formulations was evaluated in a greenhouse. The experiment was a 3 × 2 × 2 × 2 factorial with three mycelium concentrations (1.3%, 2.6%, and 3.9%), two pellet application rates (0.4 g and 1.0 g/100 g soil), two sizes of pellets (large, 8.3 mg/pellet; and small, 2.3 mg/pellet), and two storage periods (0 and 90 days after formulation). Only one control with no fungus was included; it was not factorialized. Each treatment was replicated five times. The 0-days-storage period test starting date was 7 March and the 90-days-storage period test starting date was 5 June. For each test date, five additional pots each received 1,500 nematode eggs plus autoclave-sterilized large pellets at the rate of 1.0 g pellets with 3.9% mycelium/100 g soil as controls. Each test date consisted of 60 pots that were completely randomized in a greenhouse.

Ten-cm-d clay pots were filled with 500 cm^3 steam-sterilized fine sandy soil (91% sand, 5% silt, 4% clay; <1% organic matter; pH 6.9) infested with nematode eggs. Eggs of the *H. glycines* race 14 isolate to be added to the soil were obtained from cysts crushed with a ground-glass homogenizer and rinsed through a 74-μm-aperture sieve to remove unbroken cysts and large pieces of cyst wall. About 1,500 eggs were added per 100 g soil (dry weight); concurrently, pellets of ARF18 were added. The nematode eggs, pellets, and soil were immediately and thoroughly mixed in a polyethylene bag. A single Lee 74 soybean seedling in the cotyledon stage was planted in each pot and the pots were placed in a greenhouse. The greenhouse was maintained at 22 ± 3 C. Plants were watered twice daily and received supplemental fluorescent light from 17:00 to 22:00.

Two months after planting (approximately two nematode generations), J2 and cysts in the soil were extracted from each pot by double sieving and centrifugal flotation (13) of all soil and roots from each pot. All cysts extracted from a pot were ground with a ground-glass tissue grinder (Pyrex 40 ml, Corning, NY), and eggs were collected on a 25-μm-aperture sieve, diluted, and counted at ×30 magnification. Nematode counts were subjected to analysis of variance.

Tests of soybean cultivars and H. glycines races: Alginate-clay pellets containing 2.6% mycelium were used in tests to determine whether the efficacy of ARF18 would vary among soybean cultivars and races of *H.*

TABLE 1. Population densities of *Heterodera glycines* on 'Hutcheson' soybean in sterilized soil treated with ARF18 applied in alginate-clay pellets differing in size, mycelium content, rate of application, and days stored before application.

Treatment[a]	Level	Juveniles/100 g soil	Cysts/100 g soil	Eggs/g soil
Mycelium biomass in pellet	1: 0%	170 ± 54	277 ± 40	229 ± 39
	2: 1.3%	534 ± 93	265 ± 27	166 ± 20
	3: 2.6%	339 ± 76	150 ± 16	89 ± 17
	4: 3.9%	72 ± 20	57 ± 9	18 ± 3
Rate of application[b]	0.4%	394 ± 72	176 ± 21	101 ± 15
	1.0%	208 ± 41	126 ± 14	72 ± 12
Size of pellet[c]	2.3 mg	379 ± 70	178 ± 22	101 ± 15
	8.3 mg	231 ± 49	127 ± 14	73 ± 13
Days of storage	0 days	207 ± 58	121 ± 20	38 ± 8
	90 days	395 ± 60	181 ± 16	134 ± 16

Data are means ± standard error of the mean. The treatments were 3 × 2 × 2 × 2 factorial and replicated five times. The 0.0 pellets were not considered part of the factorial because only 10 replications of this treatment were included in each storage period, whereas 40 replications of each of the other mycelium concentrations were included in each planting date.

[a] Pellets, 1.3%, 2.6%, and 3.9% mycelium concentration (w/w), are equivalent to 250 ml, 500 ml, and 750 ml of spent medium/liter alginate-mycelium-clay mixture, respectively. The 0% level of pellets contained 3.9% mycelium but had been autoclaved.

[b] Rate of application was based on soil weight.

[c] Small pellets (2.3 mg/pellet) were obtained by grinding large pellets (8.3 mg/pellet).

glycines. Large pellets at 1.0 g and eggs of *H. glycines* at 1,500 per 100 g soil (dry equivalent) were added to soil and mixed. Soil, pot size, and experimental procedures were the same as for the formulation test.

In one test, 'Hutcheson' soybean (susceptible to *H. glycines*) was grown in soil infested with an isolate of *H. glycines* race 2, 3, 4, 9, 12, or 14. In another test, the nine soybean cultivars—Pioneer 9391 (maturity group IV), Stoneville FFR 561 (V), Hutcheson (V), Pioneer 9591 (V), Davis (VI), Lee 74 (VI), Lee No-nod (VI), Sharkey (VI), and Tracy-M (VI)—were grown separately in soil infested with an isolate of race 14 of *H. glycines*. In both the race and the cultivar tests, treatments were replicated five times with separate controls that received autoclave-sterilized pellets at the rate of 1.0 g/100 g soil. All pots were completely randomized in a greenhouse. Means were compared by LSD.

TABLE 2. Statistical comparisons of population densities of *Heterodera glycines* on 'Hutcheson' soybean in sterilized soil treated with ARF18 applied in alginate pellets differing in size, mycelium content, rate of application, and days stored before application.

Factors	J2	Cysts	Eggs
Mycelium level in pellets			
Contrast			
0.0 vs 1.3	*	NS	NS
0.0 vs 2.6	NS	***	***
0.0 vs 3.9	NS	***	***
1.3 vs 2.6	**	***	***
1.3 vs 3.9	***	***	***
2.6 vs 3.9	***	***	***
Mycelium (M)	***	***	***
Rate (R)	**	**	*
Size (S)	**	**	**
Date (D)	**	**	***
M × R	*	*	***
M × S	*	***	***
M × D	NS	NS	***
R × S	**	NS	NS
R × D	NS	NS	**
S × D	**	*	*
M × R × S	NS	NS	NS
M × R × D	NS	NS	NS
M × S × D	**	*	NS
R × S × D	NS	NS	NS
M × R × S × D	NS	NS	NS
CV (%)	109.4	59.3	69.0

The treatments were 3 × 2 × 2 × 2 factorial and replicated five times. *,**,*** = significant at $P < 0.05$, 0.01, and 0.001, respectively, based on ANOVA.

RESULTS

Effect of pellet formulations on H. glycines: In general, control pots had larger numbers of nematodes than did fungus-treated pots ($P = 0.001$) (Table 1). Fungus-treated pots had fewer cysts (Tables 1, 2), and many cysts were infected by the fungus. Infected cysts were small (0.3–0.5 mm), grayish yellow, and frequently had masses of hyphae (0.1–0.2 mm) affixed to the cyst wall. Infected cysts that produced eggs contained fewer eggs than healthy cysts.

There were significant two-way interactions between treatments except between application rate and size of pellet (Tables 1, 2). Pellets containing more mycelium were more effective in suppressing egg production and maintained infectivity better in storage (Tables 1, 2). For example, pellets with 3.9% mycelium stored 90 days suppressed egg production by 92%, followed by 62% suppression with 2.6% mycelium pellets, and 26% suppression with 1.3% mycelium pellets. The relationship between the four mycelium concentrations was linear according to a single-degree-of-freedom comparison ($P < 0.0001$). Freshly made 1.3% mycelium pellets were effective in suppressing egg production only when the large size was applied (Tables 1, 2).

Soybean cultivars and H. glycines races: The efficacy of the pellets was not affected by soybean cultivar or race of the nematode ($P = 0.05$) (Figs. 1, 2). In the absence of the fungus, egg density averaged 230 eggs/g soil on the nine soybean cultivars, whereas in fungus-treated soil, egg density averaged 7 eggs/g soil, a reduction of 97% (Fig. 1). With the six race isolates of *H. glycines*, average egg density was 221 eggs/g soil in control pots compared with 11 eggs/g soil in fungus-treated pots, a reduction of 95% (Fig. 2).

DISCUSSION

Mycelium of ARF18 formulated in alginate-clay pellets suppressed reproduction

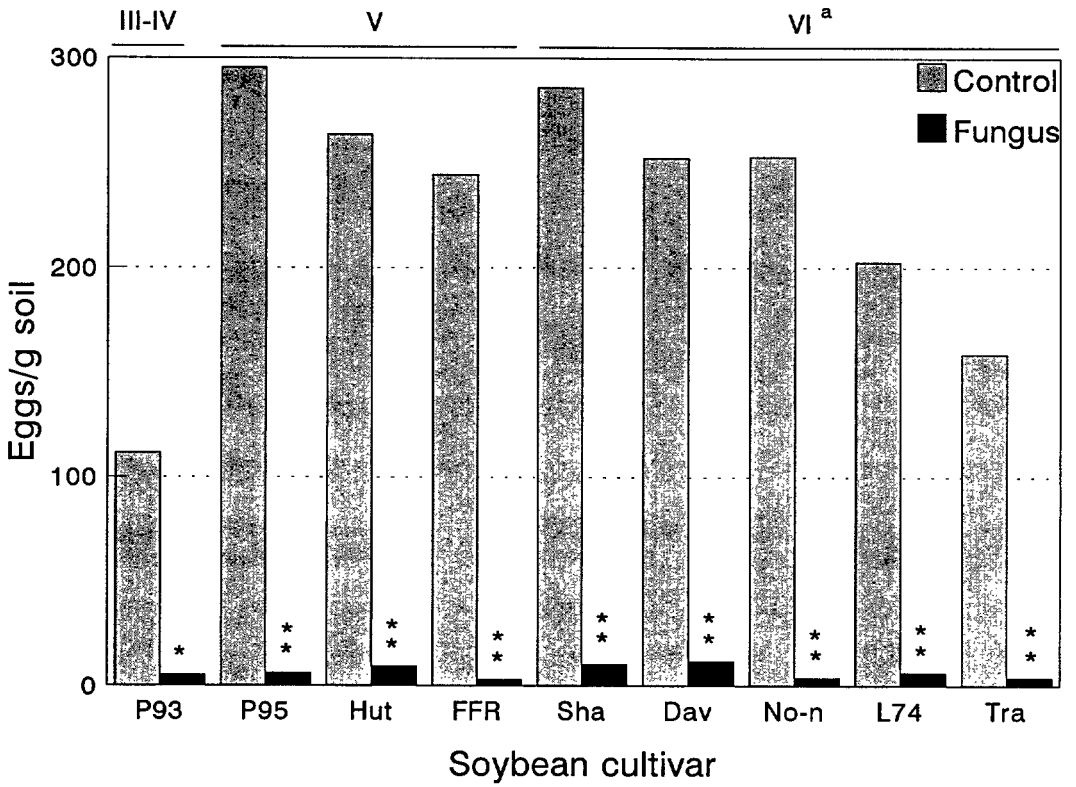

FIG. 1. Effect of ARF18 in alginate-clay pellets on numbers of *Heterodera glycines* race 14 eggs on nine soybean cultivars. Fungus treatments received 1,500 eggs and 1.0 g freshly made 2.6% mycelium pellets/100 g soil. Controls received 1,500 eggs and 1.0 g autoclave-sterilized pellets/100 g soil. Cultivars, left to right: Pioneer 9391 (Maturity Group III–IV), Pioneer 9591 (V), Hutcheson (V), Stoneville FFR 561 (V), Sharkey (VI), Davis (VI), Lee No-nodule (VI), Lee 74 (VI), and Tracy-M (VI). *, **: Control vs fungus-treated significantly different at 5% and 1% levels, respectively. The number of replications per treatment was five.

by *H. glycines*. Pellets of 3.9% mycelium biomass concentration were 3–5 times more effective than pellets of 1.3% or 2.6% mycelium concentration. Hyphae of ARF18 must make physical contact with nematodes to establish a parasitic relationship. Because equal numbers of nematodes were thoroughly mixed with the soil in all pots, the nematode host densities were the same in all treatments. Pellets made with 3.9% mycelium had 1.5–3.0 times higher mycelial biomass per pellet than pellets of 1.3 or 2.6% mycelium concentrations, so the former probably produced more hyphae per pellet and had greater probability of making contact with nematodes. Similarly, small amounts of mycelium biomass (3.0–7.5 g wet weight) of *Trichoderma* spp. and *Gliocladium virens* in pellets were less effective than large amounts (15–30 g) in suppressing *Rhizoctonia solani* in field soil (8).

The lower efficacy of small pellets, especially freshly made 1.3% and 2.6% mycelium pellets, was unexpected. Knudsen et al. (6) suggested that large numbers of small pellets may be more efficient than smaller numbers of large pellets for controlling aphids with the conidia of *Beauveria bassiana*. The reason for the lower efficacy of small pellets in our study is unclear. However, when sodium alginate solution is dropped into a solution containing calcium ions, the alginate molecules on the surface of the drops react with the calcium ions, polymerizing to form a skin by cation-dependent cross-linkages. The polymer structure is remarkably stable when stored dry and protects enclosed microorganisms from solar radiation, high

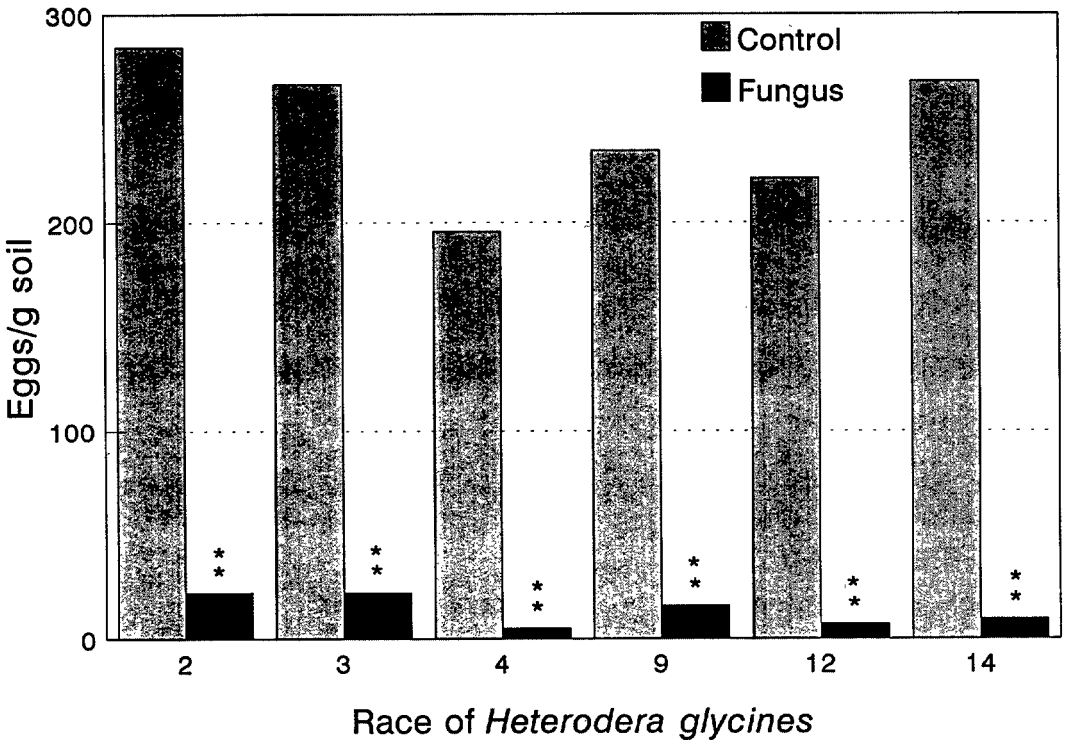

FIG. 2. Effect of ARF18 in alginate-clay pellets on numbers of *Heterodera glycines* eggs from isolates representing six races on Hutcheson soybean. Fungus treatments received 1,500 eggs and 1.0 g freshly made 2.6% mycelium pellets/100 g soil. Controls received 1,500 eggs and 1.0 g autoclave-sterilized pellets/100 g soil.

temperature (12), and change of moisture (10). Because complete polymerization (to the center of the drop) takes a minimum of 45 minutes (9) and pellets in our tests were cured about 10 minutes, our pellets may not have been polymerized to the center of the pellet. In addition, small pellets were produced by grinding original pellets, which would have broken the polymerized outer skin. The lower efficacy of small pellets may reflect the importance of the alginate polymer structure for protection of enclosed microorganisms. The rate of biodegradation of pellets depends on soil microflora, mainly bacteria. For example, alginate pellets of *Trichoderma harzianum* dipped in polyethylene glycol, which could protect pellets from the influence of soil microflora, show greater proliferation of hyphyae in soil than untreated pellets or pellets treated with water (7).

An average suppression of 96% (ranges of 86–99%) in tests with soybean cultivars and races of *H. glycines* indicates that ARF18 is nonspecific at least with regard to soybean cultivar and *H. glycines* race. Biological control organisms often are crop- and soil-specific because of the close relationship between the biological control agent, the rhizosphere, and soil factors (2). Sometimes host roots play important roles in the interaction of antagonists (1) through root exudates and their influence on the rhizosphere microfauna. In our experiment, however, the efficacy of ARF18 was similar among soybean cultivars, probably because they were all susceptible to *H. glycines*.

The alginate-clay-mycelium pellet formulation of ARF18 reduced reproduction of *H. glycines* in greenhouse studies. The efficacy of pellets was largely influenced by mycelium concentration in the pellet but not influenced by either soybean cultivar or race of *H. glycines*. The rates of pelletized fungus used in these tests were very

high. Therefore, if ARF18 is to be a useful biological control agent for *H. glycines,* development of formulations that are more efficient at lower concentrations is crucial.

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