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# Techniques for Isolation and Evaluation of Fungal Parasites of *Heterodera glycines*[1]

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*Abstract:* A facultative fungal parasite, Arkansas Fungus 18 (ARF18), was isolated from at least 10 different sources of *Heterodera glycines* in different areas in the United States. Techniques used for isolation and in vitro evaluation of fungal parasites of *H. glycines* were described. Successful isolation of probable egg parasites depends on isolation from individual eggs. Selective isolation and a simple, yet definitive, screening system can increase the possibility of identifying effective biocontrol agents.
*Key words:* ARF18, biocontrol, egg parasite, *Heterodera glycines,* isolation, soybean cyst nematode, nematode suppressive soil, technique.

The soybean cyst nematode, *Heterodera glycines*, is one of the most important pests of soybean (*Glycine max*) throughout the world (17). *Heterodera glycines* is difficult to control by conventional methods such as resistant cultivars, crop rotations, and nematicides. Thus, biocontrol is a potentially attractive alternative.

Despite a few successful cases (4,5), in general, results of pot and field experiments with predatory fungi for the control of cyst nematodes have been negative or inconclusive (6,7,19). Predatory fungi may kill second-stage juveniles (J2) of cyst nematodes in the soil but usually do not prevent population increases because the reproductive capacity of cyst nematodes is prodigious. The nonspecific feeding habits by most predatory fungi also appears to be a serious drawback.

A promising approach to the biocontrol of cyst nematodes is the use of parasites and predators attacking females and eggs in order to prevent reproduction and replenishment of juveniles (20). In the United States, surveys of fungi colonizing females and cysts of *H. glycines* identified approximately 150 species of fungi (3,10,14–16). The most frequently isolated fungi included species of *Exophiala, Fusarium, Gliocladium, Neocosmospora, Paecilomyces, Phoma, Stagonospora,* and *Verticillium* (3). Most of those fungi grew from intact cysts or females, surface-disinfested and placed on water agar. Eggs within a cyst are surrounded by a mucilage formed from the breakdown of the female's internal organs (9). This mucilage is accessible to soil microorganisms through natural openings. Thus, isolation of microorganisms from intact cysts does not exclude saprophytes.

Successful isolation of probable egg parasites depends on isolation from individual eggs. Along with isolation, a simple yet definitive screening system including both in vitro and in vivo assays must be used. The purpose of this paper is to describe the methods used in isolation and evaluation of a facultative fungal parasite of *H. glycines*, Arkansas Fungus 18 (ARF18).

## Materials and Methods

A nonsporulating Ascomycetous fungus, ARF18, was first isolated from an area in Arkansas where populations of *H. glycines* had declined from 140 eggs/g soil in November to 4 eggs/g soil the next July, a reduction of 97% (Kim, unpubl.), whereas the percentage reduction of eggs in another area under comparable conditions was 41.5–66.4% (8). Application of 37% formalin solution to the soil at the fungicidal but not nematicidal rate of 0.4 ml/100 g soil (21) resulted in 23- to 60-fold increases in the number of nematode eggs compared with untreated soil in a greenhouse test.

*Isolation from eggs of H. glycines:* Cysts were extracted by the wet-sieving and cen-

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trifugal sugar flotation method (18). The extracted cysts were ground in a 40-ml capacity glass tissue grinder to release the eggs. The suspension of eggs and broken cyst wall was poured into two 15-ml conical centrifuge tubes and centrifuged for 4 minutes at 1,800 g. A conical centrifuge tube was used because fewer eggs were lost. The supernatant was discarded and 10 ml sucrose solution (sp. gr. 1.18) was poured into each tube to resuspend the pellet. Tubes were centrifuged for 2.5 minutes at 1,800 g to separate eggs from broken cyst walls and other debris. The supernatant was poured onto a 25-μm-pore sieve, rinsed thoroughly with tap water to remove the sucrose solution, washed into sterile centrifuge tubes, and centrifuged at 1,800 g for 2.5 minutes. The supernatant was discarded.

Ten ml freshly made 0.5% NaOCl was poured into the centrifuge tube containing the pellet of eggs to surface-disinfest the eggs. The centrifuge tube was shaken vigorously for 10 seconds, allowed to stand for 30 seconds, and centrifuged for 2 minutes at 1,800 g. The supernatant was discarded and residue was rinsed twice in sterile water by repeating the shaking and centrifugation. After the final rinse, the supernatant was discarded.

A few drops of sterile water were added to the centrifuge tube containing the pellet of surface-disinfested eggs to obtain the desired egg density (ca. 2,500 eggs/ml water). With a sterile pipet, eggs (500 eggs/60 × 15-mm Petri dish) were distributed evenly over the surface of 1.5% water agar supplemented with 12.5 mg of chlortetracycline-HCL and 300 mg of streptomycin sulfate per liter. The plates were incubated at 21 ± 2 C for 3–7 days. Each plate was inspected daily at ×30 magnification with a stereoscopic microscope. Fungal hyphae that grew from eggs were transferred aseptically to cornmeal agar and were incubated at 24 C. Dishes that were overgrown with fungi or eggs with multiple fungal colonies were discarded.

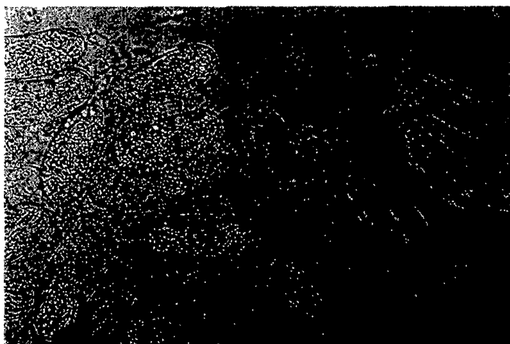

FIG. 1. Infected (left) and healthy (right) eggs are identified with the aid of modified lactoglycerol solution, 85% lactic acid:99.5% glycerol:distilled water = 2:2:1. Infected eggs absorb lactoglycerol solution readily and become clearer within a minute, whereas uninfected eggs with lipid layer intact are resistant to lactoglycerol solution and remain unchanged in appearance (darker) for 15 minutes. Bar = 37 μm.

*Isolation from infected cysts of H. glycines:* Cysts infested by ARF18 (i.e., cysts that contain infected eggs) are distinct (11–13): mycelial clusters (0.02 mm × 0.04 mm) are attached to the cuticles of small, pale-yellow cysts. When broken open, infected cysts fracture and release few eggs, whereas healthy cysts burst and release many eggs. Isolation of ARF18 can be made directly from the infested cysts.

All procedures were performed under aseptic conditions with the aid of a stereoscopic microscope. Cysts were isolated as described previously. Infested cysts (20–30 cysts) were picked directly from the sample in water, and care was taken not to break the cyst cuticle. The cysts were transferred into 0.3 ml 0.5% NaOCl in a watch glass and were surface-disinfested for 5 to 10 minutes, after which the NaOCl was replaced with 0.3 ml sterile water with a syringe. Each cyst was broken open with forceps and needle to release the eggs. The eggs and broken cyst walls were allowed to settle for ca. 2 minutes. The water was carefully removed with a syringe (0.5 $cm^3$ capacity) and was replaced with 0.3 ml 0.5% NaOCl to surface-disinfest the eggs. After 1 to 3 minutes, the NaOCl solution was replaced with sterile water with a syringe. The eggs were rinsed twice with sterile water and transferred to 1.5% water agar supplemented with antibiotics and incubated as described above.

*Test of pathogenicity:* Cysts full of eggs and free of parasites should be used. Pathogenicity tests can sometimes be improved by surface-disinfestation of cysts in 0.5% NaOCl for 5 minutes because contaminants, mainly bacteria and fungi, may interfere with the test fungus (pers. obs.).

A 4-mm-d plug cut from an actively growing fungal colony was placed colony-side-up in the center of a Petri dish containing 1.5% water agar. Five cysts of *H. glycines* that had been surface-disinfested were placed directly on top of the agar plug. The dish was stored at 21 ± 2 C (room temperature) for 10 days in a plastic bag to prevent drying, after which each cyst was picked from the agar plug and placed in a drop of modified lactoglycerol solution (lactic acid [85%]: glycerol [99.5%]: distilled water = 2:2:1) on a glass slide. The cyst was crushed in lactoglycerol solution and the contents were spread for examination of eggs. Infected and dead eggs became clearer within a minute in lactoglycerol solution, whereas uninfected eggs remained unchanged in appearance (darker) for 15 minutes (Kim, unpubl.). The eggs were observed at ×30 magnification with a stereoscopic microscope with the source of the illumination set at a low horizontal angle. The low angle of light facilitates discrimination of the translucent infected eggs from the darker uninfected eggs.

## Results and Discussion

Usually, ARF18 grew from eggs in 3 days. Both methods have been successfully used to isolate ARF18 from eggs of *H. glycines* from at least 10 different sources of cysts in different areas in the United States. An advantage of the method described in this paper is that fungi growing from eggs can be spotted and transferred with relative ease. Whenever possible, use fewer eggs per dish to facilitate subsequent observation and separation of hyphae; this is especially helpful when fast-growing fungi (generally contaminants) are present. The water agar plates should be poured several days before the addition of eggs. This allows excess water to be absorbed and makes observation and isolation easier because it reduces the activity of hatched juveniles, minimizing the spread of contaminants (1). In some cases, zoosporic fungi (Mastigomycotina) were favored by fresh agar or free water.

When cysts were old or the number of cysts were fewer than 1 cyst/g soil, the nematode population was increased in pots in a greenhouse before isolation. In general, ARF18 was readily isolated from cysts after culture in a greenhouse for 2–3 months. The close proximity of abundant nematodes under a controlled environment may favor ARF18. Isolation from infested cysts was easier and more efficient because infected eggs were concentrated.

Once a fungus is isolated in pure culture, its pathogenicity must be evaluated for direct use as an agent of biocontrol. Perhaps the simplest method is to place nematodes (eggs, egg masses, or cysts) on fungal cultures and measure the efficacy of the fungus by counting the numbers of infected eggs. In our procedure to evaluate pathogenicity of potential biocontrol agents, whole cysts are preferred as sources of inoculum because: i) cysts are the major inoculum source; ii) the cyst is the nematode stage the fungus is more likely to encounter in the soil; and iii) cysts are known to be resistant to adverse conditions.

The difference in absorption of lactoglycerol solution between infected and healthy eggs apparently was caused by the fact that healthy nematode eggs with lipid layer intact are impermeable (2).

This paper has outlined some important considerations involved in isolating and evaluating fungal parasites of nematodes, by examining the procedures used for the fungal parasite ARF18 of *H. glycines*. More efficient methodologies for the isolation and screening of nematode parasites should increase the likelihood of success in developing biocontrol agents.

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