



Effect of a New Growth Medium on *Mycoleptodiscus terrestris* (Gerd.) Ostazeski

by Judy F. Shearer

PURPOSE: One of the required steps in the development of *Mycoleptodiscus terrestris* (Gerd.) Ostazeski as a bioherbicide for *Hydrilla verticillata* (L.f.) Royle was to find a growth medium for the fungus that was inexpensive and adaptable to liquid culture fermentation processes. Because changes in the growth medium can influence fungal characteristics including virulence, it was subsequently necessary to test the effect of those changes on living plant material. This technical note describes aquarium experiments conducted to evaluate efficacy of wet and dry inoculum against hydrilla.

BACKGROUND: During the late 1980's a survey of hydrilla populations in the southern United States resulted in the isolation of an indigenous pathogen that was capable of significantly reducing hydrilla biomass in greenhouse and field trials (Joye 1990; Joye and Cofrancesco 1991). Originally reported to be a strain of *Macrophomina phaseolina* (Tassi) Goid. (Joye 1990; Joye and Paul 1991), the fungal isolate was later determined to be *Mycoleptodiscus terrestris* (Shearer 1993). Subsequent hydrilla collections in Texas during the 1990's resulted in the isolation of an additional *M. terrestris* strain. It is this isolate that has been used in more recent tests and has demonstrated excellent potential as an inundative or bioherbicidal agent that can be applied alone or in combination with chemical herbicides for management of hydrilla (Shearer 1996, 1998; Netherland and Shearer 1996; Nelson, Shearer, and Netherland 1998).

The inundative or bioherbicide approach involves the release of an agent, usually a plant pathogen, in sufficient numbers to control a target weed (Harley and Forno 1992). The pathogen is applied to specific areas in predetermined volumes and dosages that achieve control of the target weed within an allotted amount of time and before economic losses are incurred (TeBeest 1993). The method resembles the use of a chemical herbicide in that the pathogen is applied as needed and must contact the host (Auld and Morin 1995). A major requirement for the success of such an approach is that the agent must be virulent or possess a potent weed-killing phytotoxin so as to consistently manage the pest host under field conditions (Jackson 1997; Charudattan 2001). Unlike classical biological control, the pathogen is not expected to survive or to provide control beyond the growing season in which it is applied.

A critical step in the commercialization of a pathogen such as *M. terrestris* into a bioherbicide is the development of liquid culture fermentation processes that are inexpensive and yield high concentrations of viable and highly effective propagules amenable to long-term storage (Jackson 1997). Toward this end, a cooperative project was initiated in June 2000 between the Engineer Research and Development Center, Environmental Laboratory (ERDC/EL), and the U.S. Department of Agriculture, Agriculture Research Service, National Center for Agriculture Utilization Research (USDA/ARS/NCAUR), to determine the feasibility of developing a liquid culture medium for *M. terrestris* that would result in the desired aforementioned attributes.

Initial studies were directed at determining defined nutritional conditions that optimized fungal growth. Factors such as carbon sources, nitrogen sources, trace metals, vitamins, carbon loading, and carbon-to-nitrogen ratios were considered in developing the new growth media because they all have been shown to influence biomass accumulation, propagule formation, and biocontrol efficacy (Jackson 1997). Several nitrogen sources found to yield excellent fungal growth were identified, and by varying the carbon-to-nitrogen ratios, biomass production was increased and stable propagules formed.

Small-scale tests conducted in the laboratory indicated that the newly developed growth media did not compromise fungal virulence and that *M. terrestris* remained efficacious on hydrilla. The purpose of the present investigation was to test efficacy of wet and dry fungal inoculum produced with cottonseed meal (Pharmamedia, Traders Inc., Memphis, TN) as the nitrogen source on rooted hydrilla plants in aquaria.

MATERIALS AND METHODS:

Organism: Stock cultures of *M. terrestris* stored as 1-mm agar plugs in 10-percent glycerol were retrieved from cryostorage. The plugs were plated onto potato dextrose agar (PDA) (Difco, Detroit, MI) plates and allowed to grow at room temperature for 2 weeks prior to initiation of liquid fermentation processes.

Preculture medium: A defined basal salts solution was used to supply the required micronutrients for growth of *M. terrestris* in liquid culture. The medium contained per liter of deionized water: KH_2PO_4 , 4.0 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.8 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 37 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 16 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid, 500 μg each; and folic acid, biotin, vitamin B₁₂, 50 μg each. Corn steep powder (Solulys, Rochette Industries, France) (15 g L⁻¹) was added as the nitrogen source, and glucose (Difco, Detroit, MI) (1 percent) was added as the carbon source. The glucose was prepared as a 20 percent w/v stock solution and autoclaved separately. The *M. terrestris* PDA plate cultures were chopped into approximate 1-mm pieces and added to the preculture medium. The precultures were incubated at 300 rpm on a gyrotory shaker (Innova 3000, New Brunswick Scientific, Edison, NJ). After 4 days of growth, abundant short hyphal fragments developed in the medium and became the source of inoculum for the production medium.

Production medium: The production medium contained the basal salts solution described above supplemented with glucose (6 percent) and cottonseed meal (45 g L⁻¹) as the carbon and nitrogen sources, respectively. A 10-ml aliquot of the preculture medium was added to each flask as hyphal inoculum. Following an 8-day incubation as described above, the fungal matrix in the flasks became a combination of microsclerotia, microsclerotia initials, and melanized hyphae. Flasks were hand-shaken frequently to inhibit mycelial growth on the flask wall.

Inoculum: The fungal matrix was used directly from the flasks as wet inoculum for the experiments. Dry inoculum was prepared by adding 5 percent w/v diatomaceous earth (HYFLO[®], Celite Corp., Lompoc, CA) to the fungal biomass in the flasks. The hyphal-microsclerotia-diatomaceous earth mixture was vacuum-filtered in a Buchner funnel using Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The filter cake was broken up, placed in

aluminum trays, and air-dried overnight in an operating biological containment hood. The moisture content of the dried preparation was determined with a moisture analyzer (Mettler Toledo, Greifensee, Switzerland). When the moisture content was 5 percent or less, the preparation was vacuum-packed (Henkelman H100, Hertogenbosch, Netherlands) and stored at 4 °C until used.

Efficacy studies: Hydrilla efficacy studies were conducted in 55-L aquaria in a greenhouse at ERDC/EL. Dioecious hydrilla was obtained from Suwannee Laboratories (Lake Park, FL). Lake sediment collected from Brown's Lake at ERDC was amended with ammonium chloride (0.5 g L⁻¹) and Esmigran (1.75 g L⁻¹). Four plastic containers (0.95 L) filled three-fourths with the lake sediment and containing five 20-cm hydrilla apical sprigs were placed in each aquarium. The aquaria were filled with a culture solution recommended for aquatic plant growth by Smart and Barko (1984) and air was gently bubbled into each aquarium using air stones (source) to provide water circulation. Plants were allowed to grow 28 days before testing was initiated.

To test the effects of wet inoculum on rooted hydrilla, treatments included application rates of 20.0, 10.0, 5.0, and 2.5 ml of *M. terrestris* fungal matrix and untreated controls. Prior to application in each aquarium, the fungal inoculum was mixed with 100 ml of water to disperse the microsclerotia. The diluted mixture was then applied to the water surface and allowed to dissipate over the plants. Each treatment was replicated three times. After 4 weeks exposure to the treatments, shoot biomass was collected from each aquarium and oven-dried at 60 °C to a constant weight.

A second aquarium study was set up as described above to test the efficacy of wet and dry inoculum. Treatments included 20.0, 10.0, and 5.0 ml of wet inoculum, 8, 16, and 32 g of dried inoculum, and untreated controls. Each treatment was replicated three times. The 2.5-ml treatment was excluded because the low rate was not efficacious in the first experiment. After 4 weeks exposure to *M. terrestris*, the shoot biomass was processed as described above. Approximate 0.05-g samples of dry material were sprinkled onto water agar plates to determine germination of *M. terrestris* microsclerotia. After 24 hr incubation at 28 °C, the plates were examined under a stereoscopic microscope, and the first 100 microsclerotia observed on each plate were scored for germination.

Data Analysis: Biomass data were statistically evaluated using analysis of variance (Statistica, StatSoft, Tulsa, OK). Mean separations were accomplished using Tukey's Honest Significant Difference test. Test of significance was conducted at $P \leq 0.05$.

RESULTS AND DISCUSSION: Plants treated with wet *M. terrestris* inoculum began showing disease symptoms 4 days after treatment with rates higher than 2.5 ml. Leaf tissue of symptomatic plants appeared chlorotic compared to the untreated controls. Within 7 days, hydrilla leaf and stem tissue was becoming flaccid and some stem disarticulation was evident. With the dry inoculum, symptom onset was delayed and chlorosis did not appear until 7 to 9 days following application. This was expected because, unlike the wet inoculum, the dry material was not in an active state of growth at the time of inoculation.

Shoot biomass was reduced by 5, 77, 97, and 99 percent by treatment rates of 2.5, 5.0, 10.0, and 20.0 ml of wet inoculum respectively in Experiment 1. All treatment concentrations greater than 2.5 ml resulted in significant reductions of shoot biomass compared to the untreated controls

(Figure 1). At the 2.5-ml rate, the number of fungal infective units coming in contact with the host was probably insufficient to initiate disease onset.

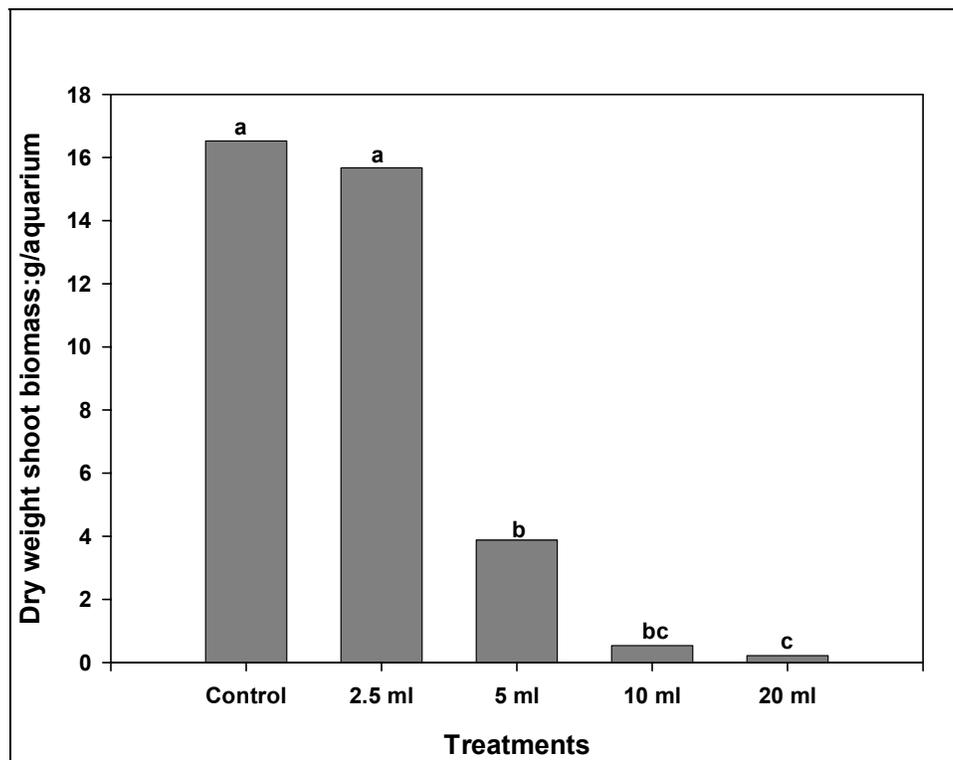


Figure 1. Mean dry weight hydrilla shoot biomass at 28 days after treatment following application of *M. terrestris* at 2.5, 5.0, 10.0, and 20.0 ml wet inoculum/aquarium. Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Tukey's Honest Significance Difference test (Experiment 1)

Similar results were obtained with wet inoculum treatments in Experiment 2. Shoot biomass was reduced 96, 98, and 100 percent by treatments of 5, 10, and 20 ml, respectively, of wet fungal inoculum (Figure 2). All three treatments significantly reduced shoot biomass compared to untreated controls but unlike experiment 1, the 5-ml treatment was as effective at reducing shoot biomass as the higher rates. Because the number of contact points between *M. terrestris* and hydrilla determines efficacy, rates less than 10 ml may result in statistically significant damage to the host but percent shoot reduction may vary. At rates of 10 ml or more, it appears that the number of infective units in the inoculum are sufficiently high that the numbers contacting the host will consistently result in a high percentage of shoot biomass reduction.

The dry inoculum treatments also significantly reduced hydrilla shoot biomass compared to untreated controls; however, the percent reduction was less than results obtained using wet inoculum (Figure 2). Only the highest rate (32 g) resulted in shoot biomass reduction (88 percent) that was not significantly different from wet inoculum treatments. The rates used in the experiment were based on laboratory bench trials using shaker flasks containing one apical hydrilla sprig submersed in 150 ml of water. Rates determined under these conditions may not translate directly to amounts that

are necessary to provide efficacy on rooted hydrilla in 55-L aquaria. Viability may also have been a factor because plating of the samples indicated that after 24 hr incubation, there was only a 72-percent germination rate of the microsclerotia. In addition, the dried material had not been formulated in any way to provide buoyancy in the water column and some of the microsclerotia precipitated to the bottom of the aquaria rather than settling on plant surfaces.

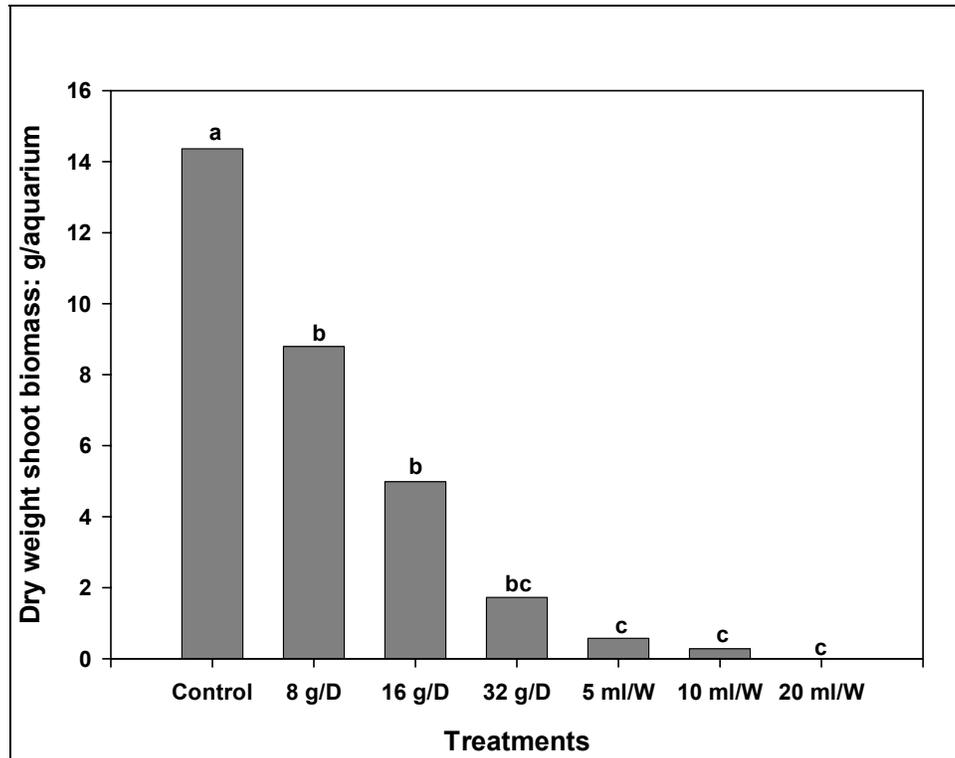


Figure 2. Mean dry weight hydrilla shoot biomass at 28 days after treatment following application of *M. terrestris* wet inoculum (W) at 5.0, 10.0, and 20 ml/aquarium and dry inoculum (D) at 8, 16, and 32 g/aquarium. Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Tukey's Honest Significance Difference test (Experiment 2)

The newly devised methods of fermenting *M. terrestris* offer several advantages compared to previous methods that used a V-8 juice-based medium. The fermentation output can now be used directly as wet inoculum without any additional processing. Previously, the V-8 medium required procedures of filtration, reconstitution, and maceration that were potentially harmful to the fungus. The number of colony-forming units (cfu) per milliliter of inoculum has been increased from a maximum of 1×10^6 cfu/ml with the V-8 medium to levels approaching 1×10^7 cfu/ml with the cottonseed-based medium. These are major improvements over earlier studies with the fungus where eight times as much inoculum was required to produce equivalent shoot reductions (Shearer 1996). Finally, the production of survival structures (microsclerotia) rather than the thin-walled hyphal units that were produced in the V-8 medium should substantially increase the shelf life of *M. terrestris*.

FUTURE WORK: Future research will focus on continuing to improve fermentation techniques for *M. terrestris* and scale up from a bench-top operation to large fermentation vessels. Each change will be monitored using appropriate flask or aquarium bioassays to ensure that fungal efficacy is not compromised. Pond trials are also planned to assess if results obtained in the laboratory are reproducible in a field situation.

POINTS OF CONTACT: For additional information, contact the author, Dr. Judy F. Shearer, (601) 634-2516, Judy.F.Shearer@erdc.usace.army.mil, or the managers of the Aquatic Plant Control Research Program, Dr. John W. Barko, (601) 634-3654, John.W.Barko@erdc.usace.army.mil, and Mr. Robert C. Gunkel, Jr., (601) 634-3722, Robert.C.Gunkel@erdc.usace.army.mil. This technical note should be cited as follows:

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