



Preliminary Testing of *Mycoleptodiscus terrestris* Formulations

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PURPOSE: This technical note reports preliminary results of bioassays of dried formulations of *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (Mt) for management of the submersed macrophyte, *Hydrilla verticillata* (L.f.) Royle (hydrilla).

BACKGROUND: As herbicide resistance becomes an increasing problem worldwide, mycoherbicides offer another option for controlling weeds. Commercializing a fungal product requires several steps starting with methods of producing abundant virulent propagules that can be used for inoculum. This can be accomplished by liquid or dry fermentation depending on the individual organism. Jackson et al. (1996) found that propagule fitness was influenced by the carbon-to-nitrogen ratio of the liquid fermentation medium. Other parameters that can be controlled during the fermentation process such as agitation and aeration rates, pH, and temperature may also affect the survival, virulence, and metabolite production of the microorganism (Charudattan 2001) as well as the type of propagule produced (Jackson 1997). In culturing *Colletotrichum truncatum* (Schw.) Andrus and Moore for management of hemp sesbania, Jackson (1997) found that carbon concentration in the medium affected the type of propagule produced. Cultures containing carbon concentrations of 4-16 g glucose per liter tended to produce thin-walled conidia whereas carbon concentrations greater than 25 g/L produced melanized aggregates called microsclerotia. Finally, the propagules that are produced must be amenable to long-term storage as dry preparations in order to commercialize them as a mycoherbicide (Jackson 1997).

Once culture methodology has been optimized, one of the major constraints to the development of efficacious and dependable mycoherbicides is an adequate formulation (Auld and Morin 1995). For terrestrial weeds the most challenging aspect to formulation is overcoming dew period requirements. While available water is obviously not a problem for mycoherbicides that are to be used on submersed plants, an aqueous environment provides other challenges to formulation, namely bouyancy and dispersal. Ideally the formulated product should be wetttable allowing it to rapidly sink on application rather than drift on the water surface. It also should have some bouyancy that keeps it suspended in the water column for sufficient time to adequately cover and attach to the surfaces of the submersed plant.

Since mycoherbicides were first developed and marketed in the United States starting in the early 1980's researchers have found that formulation supplies additional attributes to the microbial product including an increase in the effectiveness and sporulation of the pathogen on the surface of the granule (Auld and Morin 1995). The type of material used in the formulation (i.e. emulsions, organosilicone surfactants, hydrophilic polymers, or alginate-, starch-, cellulose-, or gluten-based encapsulation) may also affect product performance (Charudattan 2001). For example, a calcium alginate formulation of Mt developed by EcoScience (Worcester, MA) was ineffective for controlling Eurasian watermilfoil in part because the round pellets failed to lodge on the dissected

leaves and most ended up on the sediment surface (Shearer 1996). Additionally, some of the materials used in formulations for terrestrial plants may not be appropriate for use in aquatic systems due to potable water or irrigation issues.

In 2000, a cooperative work effort was undertaken between the U.S. Army Corps of Engineers, Engineer Research and Development Center Environmental Laboratory (ERDC-EL), Vicksburg, MS and the United States Department of Agriculture, Agriculture Research Service, National Center for Agriculture Utilization Research (USDA/ARS/NCAUR) Peoria, IL. The effort focused on developing fermentation methods for the biocontrol fungus, Mt, for management of hydrilla. The work resulted in issuance of Patent # 6,569,807 "Mycoherbicide compositions and methods of preparing and using the same" (Shearer and Jackson 2003). The defined medium that was developed induced the fungus to produce stable melanized structures called microsclerotia in liquid fermentation. The microsclerotia were harvested after 4 days by adding diatomaceous earth to the medium to facilitate separation of the melanized propagules. The medium was then vacuum filtered to remove the spent liquid of the culture broth. The harvested microsclerotia were ground and dried to approximately 5-percent moisture (Shearer and Jackson 2006). Applied at 0.05 g/L, the dried granules could reduce hydrilla shoot biomass up to 83 percent (unpublished results). Because other studies had shown that efficacy could be enhanced through formulation (Charudattan 2001), preliminary efforts were undertaken to formulate the dried microsclerotia. This report describes how Mt efficacy on hydrilla can be enhanced by coating the granules of dried Mt with two different polymers.

MATERIALS AND METHODS:

Fungal Fermentation. Microsclerotia were produced in a 100-L fermentation unit in a liquid culture medium developed by Shearer and Jackson (2006). The microsclerotia were harvested with a vacuum filtration drum that removed the liquid part of the spent medium and impinged the microsclerotia onto diatomaceous earth (HYFLO, Celite Corp., Lompoc, CA) that had been layered onto the drum. As the drum turned, a cutting blade sliced the microsclerotia/diatomaceous earth mixture into thin layers. The layers were cut into strips approximately 6 cm wide by 25 cm long and fed into a milling apparatus (Quadro, Ontario, Canada). As the strips entered the mill a rotating impeller forced them onto a screen surface where they were granulated and all granules $\leq 1397\mu$ tangentially passed through the screen openings and were collected on large aluminum trays. The granules were distributed into a thin layer and allowed to air dry overnight. When the moisture content was between 5 and 10 percent, the granules were vacuum packed into plastic bags and stored at 4° C until needed.

Fungal Formulation. The dry granules were formulated by mixing them with either 5-percent carboxymethyl cellulose (CMC) or 4-percent gum tragacanth (GT), polymers that are used in the formulation of many products from cosmetics and toothpaste to jellies and salad dressings. The granules were again passed through the milling apparatus, and collected on aluminum trays. After they had dried to a moisture content between 5 and 10 percent, they were vacuum packed and stored at 4° C until needed.

Aquarium Studies. Thirty 55-L aquariums (0.9 m tall by 0.09 m²) were filled with a water-based culture solution (Smart and Barko 1984). Lake sediment collected from Brown's Lake at the ERDC was amended with ammonium chloride (0.5 g/L) and Esmigran (1.7 g/L). Four plastic cups (0.95 L) filled three-fourths with amended lake sediment were planted with five, 20-cm apical cuttings from dioecious hydrilla, overlain with silica-sand, and placed in each aquarium. The plants were allowed to grow 28 days, by which time they had formed surface mats.

Formulated and unformulated dry microsclerotia were weighed and sprinkled over the water surface of the aquariums and allowed to naturally dissipate onto plant surfaces. Treatments included 0.02, 0.04, and 0.06 g/L dry unformulated Mt, Mt + GT, and Mt + CMC, and untreated controls. Each treatment was replicated three times. One month post inoculation, shoot biomass was harvested, dried to a constant weight at 60° C and weighed.

Flask Studies. After eight months in storage, a flask study was initiated to evaluate fungal viability and virulence of the unformulated dry Mt, Mt + GT, and Mt + CMC. Only a few grams of formulated material had been stored and sufficient quantities were not available to repeat the aquarium experiment; therefore, a flask study was initiated. Erlenmeyer flasks (250 ml) were filled with 150 ml of sterile water. One hydrilla apical sprig (20 cm long) was added to each flask. The hydrilla was inoculated with formulated and unformulated Mt in 12-mg aliquots. Each treatment was replicated five times. The flasks were placed on a laboratory benchtop under ambient temperature and light. After 2 weeks, the hydrilla in the flasks was assessed for disease using the following rating scheme:

- 0 = no disease, plant tips green and healthy
- 1 = slight chlorosis
- 2 = general chlorosis of leaves and stems
- 3 = leaves flaccid and stems fragmenting
- 4 = total shoot collapse.

Statistics. Analysis of variance (ANOVA) (Statistica Version 7, StatSoft, Tulsa, OK) was used for statistical treatment of data. Mean separations were accomplished using Tukey's Honest Significant Difference (HSD) test. Test of significance was conducted at P = 0.05.

RESULTS AND DISCUSSION: Within one week post-inoculation with Mt, disease symptoms were evident on hydrilla in the aquariums as slight chlorosis and bleaching of leaves. Over the next few days, disease severity increased as chlorosis became more widespread. Soon leaves and stems appeared flaccid, fragmentation was evident, and the plant canopy started collapsing. By two weeks post-inoculation, the diseased shoot biomass had collapsed to the bottom of the aquariums. Most tissues completely disintegrated when plants were severely damaged. All treatments using both unformulated and formulated Mt significantly reduced hydrilla shoot biomass compared to untreated controls at four weeks post-inoculation ($F(9,20) = 31.504, p = 0.000$) (Figure 1).

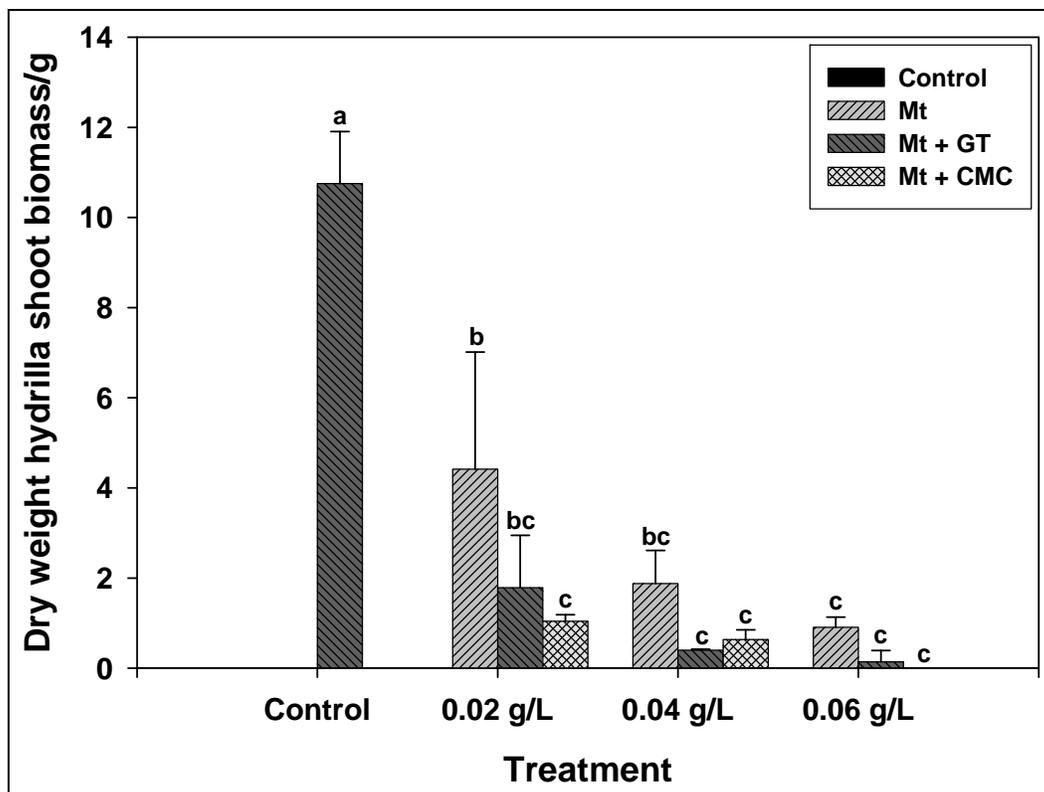


Figure 1. Response of hydrilla shoot biomass 4 weeks after treatment with unformulated (Mt) and formulated (Mt + GT, Mt + CMC) *Mycocleptodiscus terrestris*. Bars above histograms represent standard errors of the mean. Bars with different letters are significantly different (P =0.05)

The performance improvement of formulated over unformulated Mt was most apparent at the lowest application rate. Although not significantly different, the treatment of Mt + GT increased efficacy at least twofold compared with unformulated Mt applied at a rate of 0.02 g/L. The Mt + CMC treatment significantly reduced shoot biomass compared to unformulated Mt applied at the same rate and produced a fourfold increase in efficacy. This has important implications for development of a mycoherbicide because lower use rates can considerably reduce an application cost and can potentially cause less damage to non-target species. At the higher application rates, addition of polymers enhanced efficacy but the differences were not as pronounced because biomass reductions in all cases were high. At the 0.06 g per liter application rate, biomass was reduced 91.6, 98.6, and 100 percent by Mt, Mt + GT, and Mt + CMC, respectively, compared to untreated controls.

Fungal viability and virulence of the formulated Mt did not appear to be compromised by eight months in storage. During the bioassay the flasks were observed frequently for disease development. Symptoms on the hydrilla sprigs progressed as described above for the rooted plants in the aquarium study. Readings were taken after two weeks rather than four weeks because tissues had totally collapsed in flasks treated with Mt + GT and Mt + CMC. No green tissue remained; thus, both had the maximum disease severity rating of 4. The unformulated Mt had a mean disease rating of 3.3. Disease development on hydrilla treated with the unformulated Mt was variable with a high disease rating of 4 and a low rating of 1.5. Considering that hydrilla was little damaged in one of the flasks

(disease rating of 1.5), reduced viability and/or virulence cannot be ruled out. However, a few other possibilities exist including the shortened duration of the study (two rather than four weeks). Second, because Mt is a contact pathogen, lack of attachment of particles to plant surfaces could result in less disease on hydrilla in one flask compared to another. Finally, unformulated Mt did not perform overall as well as formulated Mt in the aquarium studies and these results from the flask study also seem to reflect those differences.

Great strides have been made in developing Mt into a mycoherbicide. Methods that were developed as a result of the cooperative work between ERDC and NCAUR have shown that product performance could be substantially enhanced through fermentation (Shearer and Jackson 2006). The current work holds great promise that additional efficacy can be achieved through formulation.

FUTURE WORK: Future research will evaluate additional formulations to determine the best one for enhancing Mt and for use in aquatic systems as well as field tests. The goal is to find the most efficacious formulation that uses the least amount of inoculum yet provides excellent hydrilla control.

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