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Dose Response Studies of *Mycoleptodiscus terrestris* Formulations on *Hydrilla verticillata*

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PURPOSE: This technical note describes the results of two greenhouse experiments that evaluated dose response rates of granular formulations of the fungal pathogen *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (*Mt*) against the noxious aquatic weed hydrilla (*Hydrilla verticillata* (L.f.) Royle). Information from the greenhouse experiments will be used to pinpoint areas where fungal formulation needs improvement and to better estimate dosage rates for field evaluation of formulated granules.

BACKGROUND: *Mt*, a fungal pathogen of hydrilla, applied as a liquid slurry has been demonstrated in laboratory, greenhouse, and field trials as a potential biological control agent for the submersed aquatic macrophyte (Joye 1990, Joye and Cofrancesco 1991, Joye and Paul 1992, Shearer 1996, 1997). As a first step in the process of considering development of the organism for commercial use, efforts were directed at the feasibility of formulating the fungus as a dry granule. In 1997, the biomanagement team at the U.S. Army Engineer Environmental Research Development Center, Waterways Experiment Station (WES), Vicksburg, MS, contracted with Trans America Product Technology, Inc. (TAPT) St. Charles, MO, to produce a prototype granular formulation of the fungus. Fungal slurry produced at WES was incorporated into a patented EPA-approved biocarrier (Biocar 405) at TAPT, dried, and milled. The granular product proved efficacious and significantly reduced hydrilla shoot biomass at 4 weeks post-application (Shearer 1998). Once it was established that it was feasible to formulate *Mt* as a granule, further development of the organism as a bioherbicide had to address aspects of formulation.

The two key phases in the formulation process, fermentation and granule production, will require optimization to ensure that the *Mt* product has the greatest possible number of infective units and remains virulent. It is during the fermentation phase of formulation that the fungus is mass-produced on an artificial medium. Both the medium and growth parameters can directly affect the viability and virulence of the pathogen. At present, the fermentation equipment, growth parameters, and culture medium produce a concentrated slurry containing 1×10^6 colony-forming units/milliliter (cfu/ml). Each unit is a mass of fungal cells that agglutinate together. The number of cfu's could potentially be increased if each mass of fungal cells could be sheared into smaller, but still viable, fractions followed by better mixing and dispersal in the biocarrier.

During granule production, the fungal slurry output from fermentation is incorporated into a biocarrier, extruded, dried, and milled to produce a granular product. Two aspects of this phase are particularly important. First, the biocarrier and any other components of the biological formulation have to be compatible with *Mt*. Secondly, viability and virulence of the fungus must be maintained at the highest possible levels during the incorporation process. It is already known that a log reduction in viability of the fungal component is to be expected during drying of the granule (Shearer 1998).

In reviewing the incorporation phase, it was determined that the physical properties of the granule was an area of formulation improvement that could be easily altered and evaluated. Because *Mt*

adhesion to hydrilla surfaces is a prerequisite to plant invasion and subsequent disease development in the host (Joye and Paul 1992), dispersal of the granule in an aqueous medium and adequate coverage on the target plant were of primary importance. On a per gram basis, an increase in plant coverage could potentially be achieved by reducing the particle size of the granule. Of concern in this approach was the effect that increased milling might have on the viability of the biological component.

Each alteration to the *Mt* product through formulation must be evaluated for its effect on the target plant. Any change has the potential to affect positively or negatively the performance of the biological component. This technical note reports the results of two experiments that evaluated granule performance on hydrilla biomass reductions as they related to changes in 1) granule particle size, and 2) granule cfu counts.

MATERIALS AND METHODS: The fermentation phase of the formulation process was completed in the biomanagement laboratory at WES. The fungal slurry was prepared by inoculating 1-L Erlenmeyer flasks containing 600 ml of Richard's V-8 juice broth (glucose, 10 g; KNO_3, 10g; $CaCO_3$ 3g; V-8 juice (Campbells), 200 ml; H_2O, 800 ml) with five plugs cut from the leading edge of actively growing *Mt* colonies. The flasks were agitated on a gyrotory shaker (New Brunswick, Edison, NJ) set at 200 rpms. After 6 days, the mycelial pellets from each flask were filtered through four layers of cheesecloth, reconstituted with 100 ml of sterile water, and comminuted in a blender for 30 sec. The resulting slurry was shipped under refrigeration to TAPT for granular incorporation.

At TAPT, the slurry was incorporated into a biocarrier, Biocar 405, extruded, dried, and milled into granular particles. In order to test the effect of granule size on efficacy, the particles were milled and sieved to yield two size ranges; 0.43mm-0.85mm and 0.25mm-0.42mm or 20-40 mesh and 40-60 mesh, respectively. To test effects of dilution, mixing, and dispersal on granular efficacy, the slurry was partitioned into two subsamples prior to incorporation into the biocarrier. One subsample remained undiluted and the second subsample was diluted 1:10 with sterile water prior to thorough mixing in the biocarrier. Following incorporation into Biocar 405, the subsamples were extruded, dried, milled, and sieved to yield 40-60 mesh granules. The final products were shipped under refrigeration to WES where they were analyzed for viability and efficacy in laboratory and greenhouse studies.

Viability of the granules was determined by serial dilution. The dilutions were plated onto Martin's agar (H_2O, 1 L; agar, 17 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; peptone, 0.5 g; dextrose, 10 g; yeast extract, 0.5 g; rose bengal, 0.05 g; streptomycin sulfate, 0.03 g) in 1-ml aliquots and incubated in the dark at 28 °C. After five days, *Mt* colonies on the plates were counted to determine cfu's of the fungus per gram of biomass.

Granule efficacy was evaluated on hydrilla plants grown in 50-L aquariums. Approximate 1-L plastic cups were filled with lake sediment to within 5 cm of the top of the cup and overlain with silica sand. Five 15-cm apical sprigs of hydrilla were planted in each cup. Four cups were placed in each 50-L aquarium containing a nutrient solution (Smart and Barko 1985). After the hydrilla plants reached the surface of the water, the formulation granules were applied. The granules were dispensed evenly over the water surface in 1,3,5,7,9,11,13,and 15-g doses and 1,3,5, and 7-g doses for experiments 1 and 2, respectively. Each treatment was replicated three times. After 4 weeks, hydrilla aboveground biomass was harvested and dried at 60 °C to a constant weight.

Data were subjected to analysis of variance (ANOVA) procedures (Sigma Stat 2.0, SPSS, Inc., Chicago, IL). LSD tests (P=0.05) were used to determine differences among means when the overall ANOVA was significant.

RESULTS AND DISCUSSION: The *Mt* slurries produced in shaker flask culture at WES and shipped to TAPT for formulation completion contained 1 x 10^6 cfu/ml. These counts were consistent with previous studies using the same techniques and broth medium (Shearer 1998, Shearer and Nelson 1999).

The additional milling necessary to produce small granule sizes did not seem to affect fungal cfu counts. Both the 20- to 40-mesh and 40- to 60-mesh granules contained 3.3 x 10^5 cfu/g. Particle size did affect the dispersal characteristics of the granules. The 40- to 60-mesh particles floated on the water surface for several minutes before they began to fall through the water column. In a field situation this characteristic could result in dry inoculum being transported well away from the site of application by simple water movement. In all likelihood, 40- to 60-mesh particles would have to be applied as a wettable powder to prevent such dispersion. In contrast, the 20- to 40-mesh particles began to disperse immediately after application. Testing under field conditions would be necessary to determine if movement away from the site of application would also be a problem for the larger 20- to 40-mesh granules.

Efficacy of the granules was not affected by particle size. There was no significant difference between the overall performance of the 20- to 40-mesh granules and the 40- to 60-mesh granules (P = 0.721). Efficacy was significantly affected by inoculation rates, however. Granules applied at rates of 3 g and higher induced significant reductions in aboveground hydrilla biomass compared to untreated controls (Figure 1). Biomass reductions of greater than 80 percent compared to untreated controls were achieved with 20- to 40-mesh granules applied at 3, 5, 7, 9, 11, 13, and 15 g and for 40- to 60-mesh granules applied at 5-, 7-, 9-, 11-, 13-, and 15-g treatments, respectively.

Shearing of the slurry in a commercial blender, followed by dilution and additional mixing during incorporation into the biocarrier, did not result in breaking up the large fungal units into smaller viable fractions. The subsamples contained 2.6 x 10^5 cfu/g and 1.6 x 10^4 cfu/g using undiluted and the 1:10 diluted slurry, respectively. Although formulation parameters were unchanged, cfu's of the dry granules produced from the undiluted slurry for experiment 2 had approximately 21 percent fewer viable units per gram than those used in experiment 1. The result of further diluting the slurry tenfold was reflected in an approximate log reduction in cfu counts of the formulated granules. Overall, neither configuration was effective in substantially reducing hydrilla aboveground biomass (Figure 2). Only one treatment, the 5-g undiluted slurry granule, resulted in a 75-percent biomass reduction, which was significantly different than the untreated controls. This is in contrast to experiment 1, where dose rates as low as 3 g resulted in a biomass reduction of 88 percent. It should be noted that this seemingly low dose rate would require excessive amounts of inoculum for field applications. Granule performance must be improved to make this potential biocontrol product competitive with currently used herbicides.

FUTURE WORK: The experiments reported herein point to the need to improve both phases of *Mt* formulation. It is critical that the number of viable fungal units in the slurry be increased well above the present 1 x 10^6 cfu/ml to maximize the potential number of infective units in the product and to offset loss of viability incurred during drying of the granules. Several options for

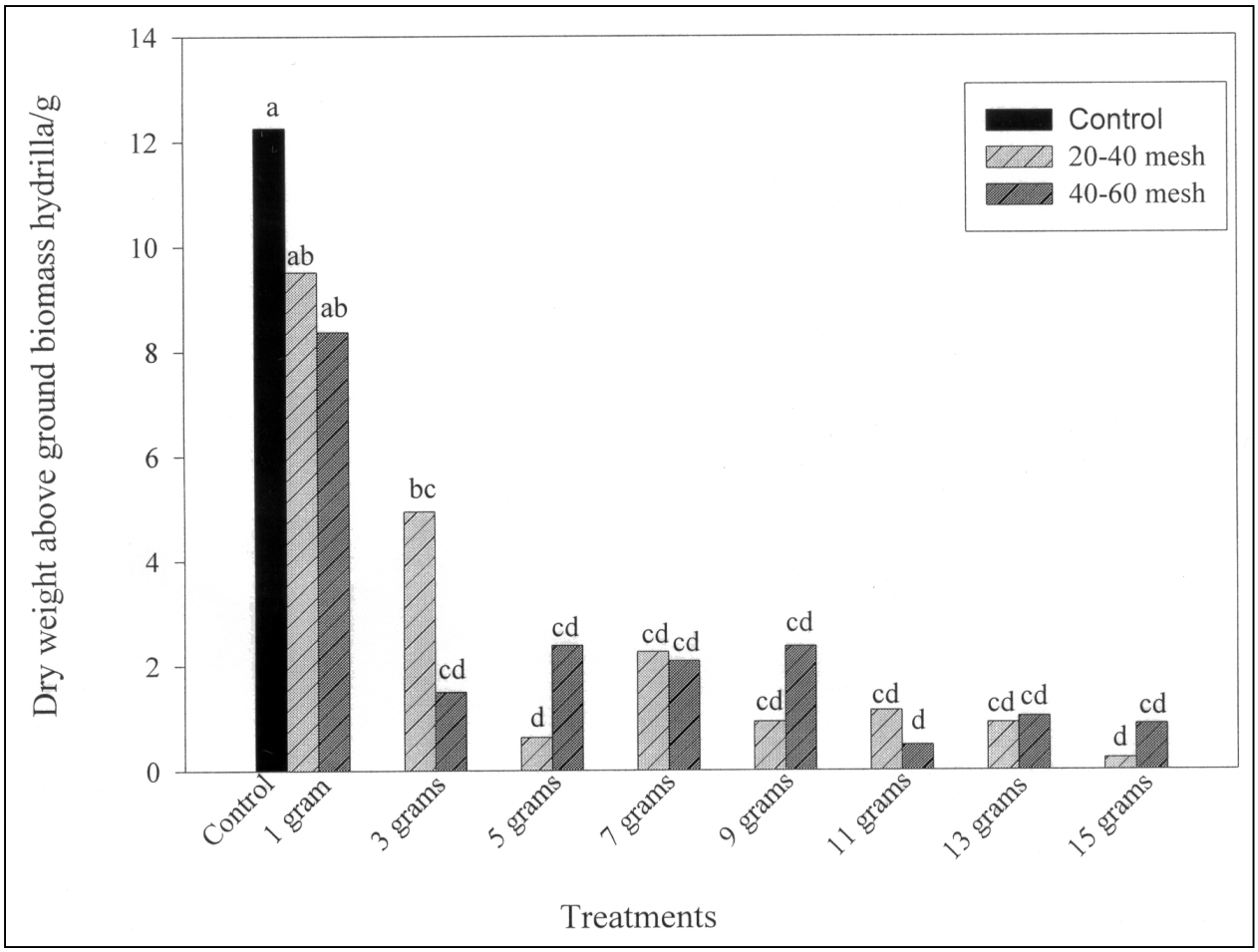

Figure 1. Mean dry weights of hydrilla aboveground biomass at 4 weeks after treatment following application of different dose rates of *Mt* formulation milled to different mesh sizes. Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Bonferroni *t*-test

improvement of the fungal component during the fermentation phase will be investigated including development of a defined growth medium, growth parameters (e.g. temperature, pH, aeration, agitation), inducement of fungal sporulation, and shearing of the mycelium following fermentation. Inability to substantially improve the fermentation aspect of formulation will in all likelihood curtail the further development of *Mt* as a bioherbicide.

Inconsistencies in efficacy of granular formulations suggest the need for changing and improving the incorporation phase of formulation development. Additional biocarriers will be evaluated to find one that offers optimum compatibility and consistent performance capabilities when combined with *Mt*. To alleviate past problems with biocarrier inconsistencies, a quality control assay must be developed that ensures reproducibility of biocarrier physical and chemical properties from lot to lot. Research efforts will also address changes in equipment and/or procedures that may help lessen viability losses in the fungal component that are presently incurred during the processes of extrusion, drying, and milling of the granule.

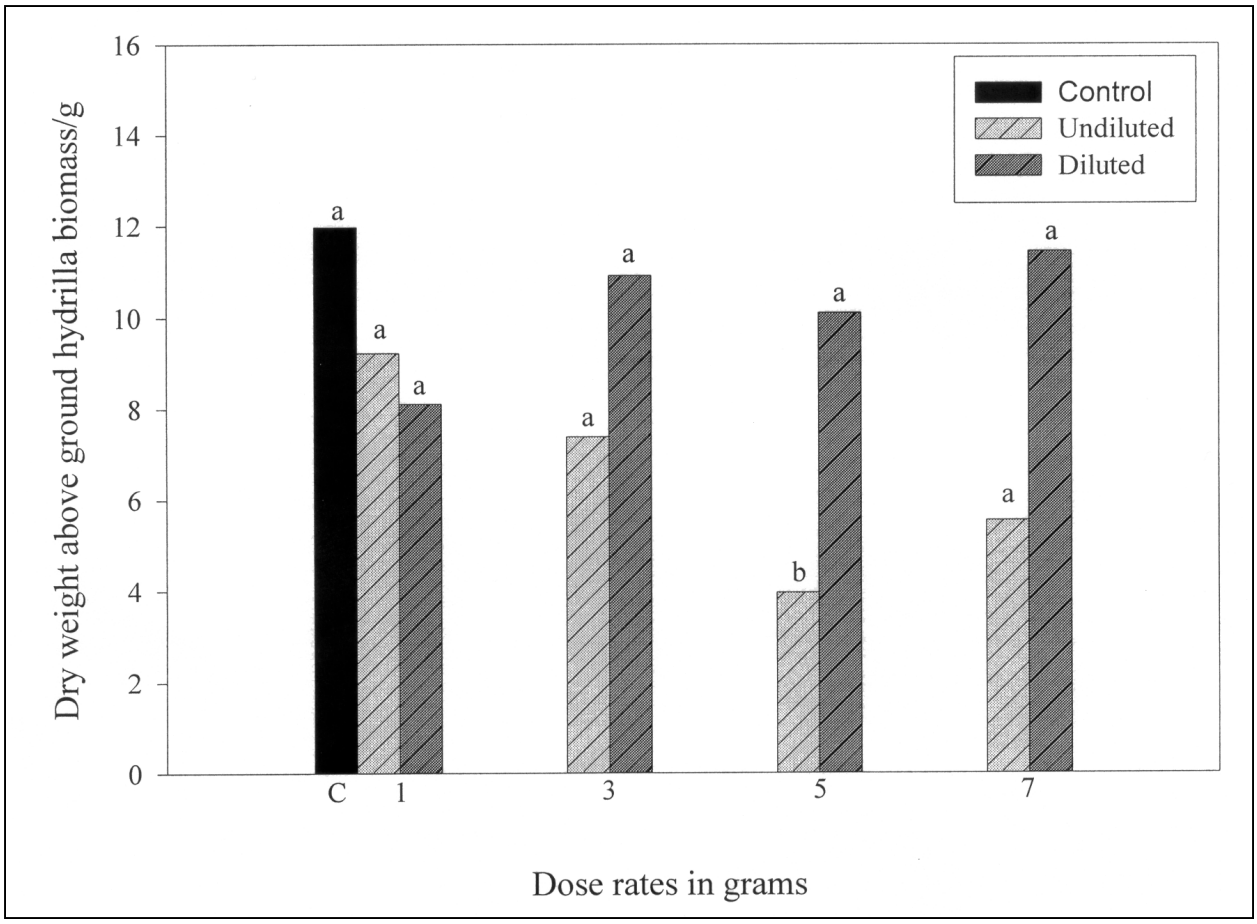

Figure 2. Mean dry weights of hydrilla aboveground biomass at 4 weeks after treatment following application of different dose rates of *Mt* formulation that was prepared with diluted and undiluted slurry. Means followed by the same letter are not significantly different at P≤ 0.05 according to Bonferroni *t*-test

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