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Biological Control of Hydrilla Using an Endemic Fungal Pathogen

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ABSTRACT

Prototype formulations of the hydrilla fungal pathogen, *Mycoleptodiscus terrestris* (Gerd.) Ostazeski, were tested for efficacy against dioecious *Hydrilla verticillata* (L.f.) Royle in test tube, column, and tank studies. The formulation was produced by Trans America Product Technology, Inc. (St. Charles, MO) by incorporating the fungus into a patented biocarrier, Biocar 405. Initial test tube studies demonstrated that both granular and caplet formulations induced disease ratings of 3 and 4 on excised hydrilla shoot tissue at two weeks post inoculation. Low, medium, and high dosage rates of the granular formulation applied to rooted hydrilla in 12 L columns reduced shoot biomass at four weeks post application by 87.7, 94.8, and 99.2% respectively compared to untreated controls. In tank studies a granular formulation reduced shoot biomass of hydrilla grown in 1700 L tanks by 97.5% at four weeks post application.

Key words: Mycoleptodiscus terrestris, Hydrilla verticillata, mycoherbicide, formulation.

INTRODUCTION

The endemic fungal pathogen, *M. terrestris* is a potential biological control agent for hydrilla as demonstrated by laboratory, greenhouse, and field trials (Joye 1990, Joye and Cofrancesco 1991, Joye and Paul 1992, Shearer 1996, Shearer 1997). Four to seven days following inoculation with the pathogen, the first disease symptoms appear as a chlorosis of hydrilla leaf tissue. Within two weeks, leaves and stems become flaccid, lyse, and float to the water surface. Observations from a histological study of the infection process suggested that enzymatic action allows pathogen ingress into the cells of the lower epidermis (Joye and Paul 1992). Following entry, the fungus colonizes host cells resulting in a disruption of cell walls and subsequent cell death.

M. terrestris makes an ideal candidate for inundative biological control because the pathogen is endemic, the disease episode is of short duration, and the fungus does not persist in hydrilla debris or plant tissues in the field (Shearer 1996). The approach also known as the mycoherbicide strategy utilizes pathogens in much the same way as chemical herbicides (TeBeest 1993). A pathogen is formulated and applied to a target host population in volumes that achieve control within an allotted period of time. That the strategy can be successful has been documented by the development of formulated *Colletotrichum gloeosporioides* f.sp. *aeschynomene* (COLLEGO) to control northern jointvetch in rice and soybeans in the southeast United States (TeBeest 1993) and formulated *Phytophthora palmivora* (DeVine) to control stranglervine in citrus groves in Florida (Charudattan 1991).

To date, formulation research on fungal biocontrol pathogens has primarily been directed toward development of mycoherbicides to control terrestrial weeds on agricultural land. One of the major considerations in formulation effectiveness has been the retention of a wetting or dew period to prevent desiccation of the fungus until infection can be established within host tissues (Hasan and Ayres 1990). While retention of fungal viability is a consideration in the development of a mycoherbicide for use on submersed macrophytes, dew period in an aquatic system is not of major concern. To be effective in an aquatic environment, the shape, size, and buoyancy of a formulated fungus may need to be adjusted to allow for dispersal and adequate coverage of the target plant in an aqueous medium.

Formulation development encompasses several steps including manipulation of the fungus, design of biocarriers, and incorporation of the fungus into the biocarrier. Biocar 405, a patented EPA approved biocarrier, developed by Trans America Product Technology, Inc. (TAPT, St. Charles, MO) was tested as a potential biocarrier for *M. terrestris*. Because the biocarrier had proved effective in dispersal of a mosquito pathogen in an aquatic habitat, it was thought that it might work equally well with the hydrilla pathogen. The performance of the prototype formulation of Biocar 405 and *M. terrestris* was evaluated in laboratory and greenhouse experiments on excised and rooted hydrilla.

MATERIALS AND METHODS

Seed cultures were prepared by plating *M. terrestris* onto potato dextrose agar plates (PDA) (Difco Laboratories, Detroit, MI). The cultures were incubated in the dark at 28 C for seven days. Plugs 4 mm in diameter were cut from the leading edge of the fungal colony. Five plugs of fungal mycelium were added to 1 L Erlenmeyer flasks, each containing 500 ml of modified Richard's V-8 juice broth (glucose, 10 g; KNO_3, 10 g; $CaCO_3$, 3 g; V-8 juice (Campbells), 200 ml; H_2O, 800 ml). The flasks were placed on a platform shaker (New Brunswick, Edison, NJ) and agitated at 200 rpms. After six days, the mycelial mat was filtered through four layers of cheesecloth, suspended in 100 ml of sterile water, and comminuted in a blender for 30 sec. Dilutions of the fungal suspensions 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,

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0.05 g; streptomycin sulfate, 0.03 g) to determine propagule density. The above procedures consistently result in a slurry with a colony forming unit count (cfu) of 1×10^6 colony forming units (cfu)/ml.

The fungal slurry was shipped overnight in an ice-cooled chest to TAPT Inc. for processing. The fungus was incorporated into the biocarrier, Biocar 405 (Biosorb, Inc., Belle Glade, FL). For preliminary evaluation of formulation performance on hydrilla, the matrix was extruded as dry granules and caplets. For testing in columns and tanks, a granular form of the formulation was used.

The number of cfu/g of formulated fungus was determined by suspending a 0.5 g subsample of dry granules in 10 ml of sterile water and plating a series of dilutions. After four days incubation at room temperature, *M. terrestris* colony counts were determined by visual examination of the plates.

Test tube studies

Sprigs of hydrilla 15 cm in length were collected from greenhouse hydrilla stock cultures, thoroughly washed in tap water, and placed in test tubes containing 60 ml of sterile water. An approximate 1 g dose (800 cfu/ml) of granules or one caplet of the formulated fungus was added to the water and allowed to dissipate over the plant material. Treatments were replicated five times. The test tube cultures were placed in a 26 C incubator set to a 12/12 light/dark cycle. After two weeks the plants were visually examined and rated for disease damage (0 = no damage; 1 = slight leaf chlorosis; 2 = general chlorosis of leaves and stems; 3 = leaves and stems chlorotic and flaccid; 4 = total plant collapse). Subsamples of the granules and caplets were kept cool and plated weekly onto Martin's agar to assess fungal viability. The subsamples were visually assessed for presence of *M. terrestris* colonies.

Column studies

Clear acrylic columns (76 cm tall by 13.7 cm wide) (12 L) were used for small-scale greenhouse testing. Thirty-two-ounce plastic cups filled three-fourths full with lake sediment amended with ammonium chloride (0.5 g/L) and Esmigran (1.75 g/L) were overlain with five cm of tap-water-washed silica sand. Three 15-cm apical sprigs of hydrilla were planted in the sediment and the cups placed in the bottom of the column. Twelve liters of nutrient solution (Smart and Barko 1985) were added to each column. The columns were aerated and maintained at 25 ± 1 C in an environmental chamber (Conviron, Pembina, ND). Plants were allowed to grow approximately four weeks before testing was initiated. Five, 10, and 20 g of formulated fungus were applied to the surface of the water. Allowing for dilution in the 12 L columns, the effective rates were approximately 42, 83, and 167 cfu/ml respectively. Low-and medium-dose treatments of the fungal slurry were applied at rates of 5 and 10 ml (400 and 800 cfu/ml) respectively. Each treatment was replicated three times. After four weeks, three stem pieces 2 cm in length were collected from plants or floating plant tissue in each column, surface sterilized in a 10% bleach solution (0.5% NaOCl) for 1 min, rinsed in sterile water, and plated onto Martin's agar to determine presence/absence of the fungus in the plant tissue. The remainder of the aboveground biomass from each column was harvested and dried to a constant weight at 60 C.

Tank studies

Tanks (160 cm in diam by 92 cm deep, approximately 1700 L) were used for testing formulated *M. terrestris* on a larger scale outdoor study. Lake sediment was amended as described above. Plastic containers (36 cm by 30 cm by 13 cm) were filled with sediment to a depth of 8 cm and overlain with 4 cm of tap-water-washed silica sand. Twenty-five apical sprigs of hydrilla 15 cm in length were planted in each container. Ten containers were placed in each of six tanks and the tanks filled with nutrient solution (Smart and Barko 1985). The plants were allowed to grow until they reached the water surface and formed a canopy. The granular formulation was dispersed in 400-g doses over the water surface of treated tanks and allowed to naturally dissipate onto hydrilla tissue. The effective application rate allowing for dilution was 23 cfu/ml. Treatments were replicated three times. After four weeks, aboveground biomass was harvested from treated and control tanks. Small subsamples of plant stem tissue 2 cm in length were taken from each sample to assay for presence/absence of the fungal component. The remaining biomass was dried to a constant weight at 60 C.

Data were analyzed using t tests and one-way analysis of variance (ANOVA). A confidence level of P = 0.05 was used to determine statistical significance. Analyses were performed with the Sigma Stat program for the Windows™ operating system 1995 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

One of the most important features required in the design of a successful formulation of *M. terrestris* is plant surface area coverage because each point of contact between a formulation particle and hydrilla tissue is a potential site of ingress by the fungal pathogen. The test tube studies indicated that the best coverage over plant surfaces was achieved using the granular formulation. As the granules absorbed water, they disintegrated into small particles that became lodged on plant surfaces. In contrast, the caplets disintegrated into fine particles that either lodged on surfaces, agglutinated in masses, or collected in the bottom of the test tube. The uneven distribution and wastage of particles eliminated the caplet from further evaluation as a potential formulation of *M. terrestris*. Another area of concern in formulation development was retention of viability and virulence. Physical processes of extruding and drying the formulated product had minimal effects on fungal virulence and viability.

Test tube studies

At two weeks post inoculation, damage on hydrilla using granules or caplets was assessed a disease rating between 3 and 4 (i.e. the hydrilla sprigs were severely chlorotic and flaccid or collapsed in the bottom of the tube). Viability was confirmed through consistent fungal retrieval from dried granules and caplets up to three months following production at which time the supply was depleted.

Table 1. Mean dry weight biomass of hydrilla shoot tissue collected 4 weeks post application with *M. terrestris* slurry or *M. terrestris* formulation.

Treatment	Control	Slurry 5 ml	Slurry 10 ml	Granules 5 g	Granules 10 g	Granules 20 g
Dry weight (g)	13.91a[1]	1.91b	0.76b	1.71b	0.73b	0.10b

[1]Values followed by a different letter are significantly different.

Table 2. Mean dry weight biomass of hydrilla shoot tissue following application of *M. terrestris* formulation.

Treatment	Control	Granules
Dry weight (g)	391.2a[1]	9.66b

[1]The mean values of the inoculated tanks were significantly different from those of the control tanks as determined by the t test.

Column studies

A fungal slurry of *M. terrestris* mycelia rated at 1×10^6 cfu/ml applied at 5 ml and 10 ml to rooted hydrilla reduced shoot tissue 86.3 and 94.5% respectively by four weeks post inoculation (Table 1). Comparable reductions in hydrilla shoot biomass, 87.7 and 94.8%, were achieved with applications of 5 g and 10 g respectively of *M. terrestris* formulation rated at 1×10^5 cfu/ml. At the highest rate of application (20 g), the formulated fungus reduced biomass by 99.2%.

M. terrestris was recovered from hydrilla shoot tissue four weeks post application with the fungal slurry and from the 5- and 10-g dose treatments of the formulation. Insufficient plant material remained in the 20-g formulation treatment for analysis.

Tank studies

By week one of the tank study, disease symptoms were apparent on hydrilla. Leaves were chlorotic and there was evidence of stem fragmentation. By week three of the study, the epidemic had waned and little hydrilla tissue remained in the treated tanks. Because the tanks were outdoors, there was some concern that insects might transmit fungal inoculum to untreated hydrilla, but at the four-week-post inoculation harvest, *M. terrestris* was not recovered from hydrilla shoot tissue collected from treated or control tanks.

The procedures used in processing and drying of the formulation granules, although potentially harmful to fungal mycelia, did not severely affect *M. terrestris* viability. Formulation of the fungal slurry rated at 1×10^6 cfu/ml resulted in an approximate log reduction in cfu counts. Four weeks post inoculation, hydrilla shoot biomass was reduced 97.5% compared to untreated controls (Table 2). The effective rate of fungal inoculum used in each tank (24 cfu/ml) was 66% lower than the effective rate of the low dose treatment of the column study (42 cfu/ml). Good coverage over plant tissues resulting from excellent dispersal characteristics of the granules meant less inoculum was required to achieve the desired biomass reductions. Each contact point of a formulation particle with hydrilla tissue was a potential site of fungal growth and subsequent invasion. Because the fungus does not ramify extensively within host tissue following invasion, multiple points of contact are necessary to promote a disease epidemic. In previous tank studies, inoculum applied in the form of a fungal slurry reduced hydrilla shoot biomass by 85% (Shearer 1994). A liquid inoculum has a tendency to remain suspended in the water lessening the potential for physical contact between pathogen and host that is necessary for cell invasion.

Biocar 405 appears to be an excellent biocarrier for the fungus *M. terrestris*. The formulation process preserves pathogen viability and virulence. The dry granule provided good coverage over hydrilla plant surfaces and should be easily adaptable for application with conventional spray equipment. Additional testing on a larger scale under field conditions will confirm if the formulation has potential as a marketable mycoherbicide for hydrilla.

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