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Aquatic Plant Control Research Program

Endemic Pathogen Biocontrol Research on Submersed Macrophytes: Status Report 1996

by Judy F. Shearer

Prepared for Headquarters, U.S. Army Corps of Engineers

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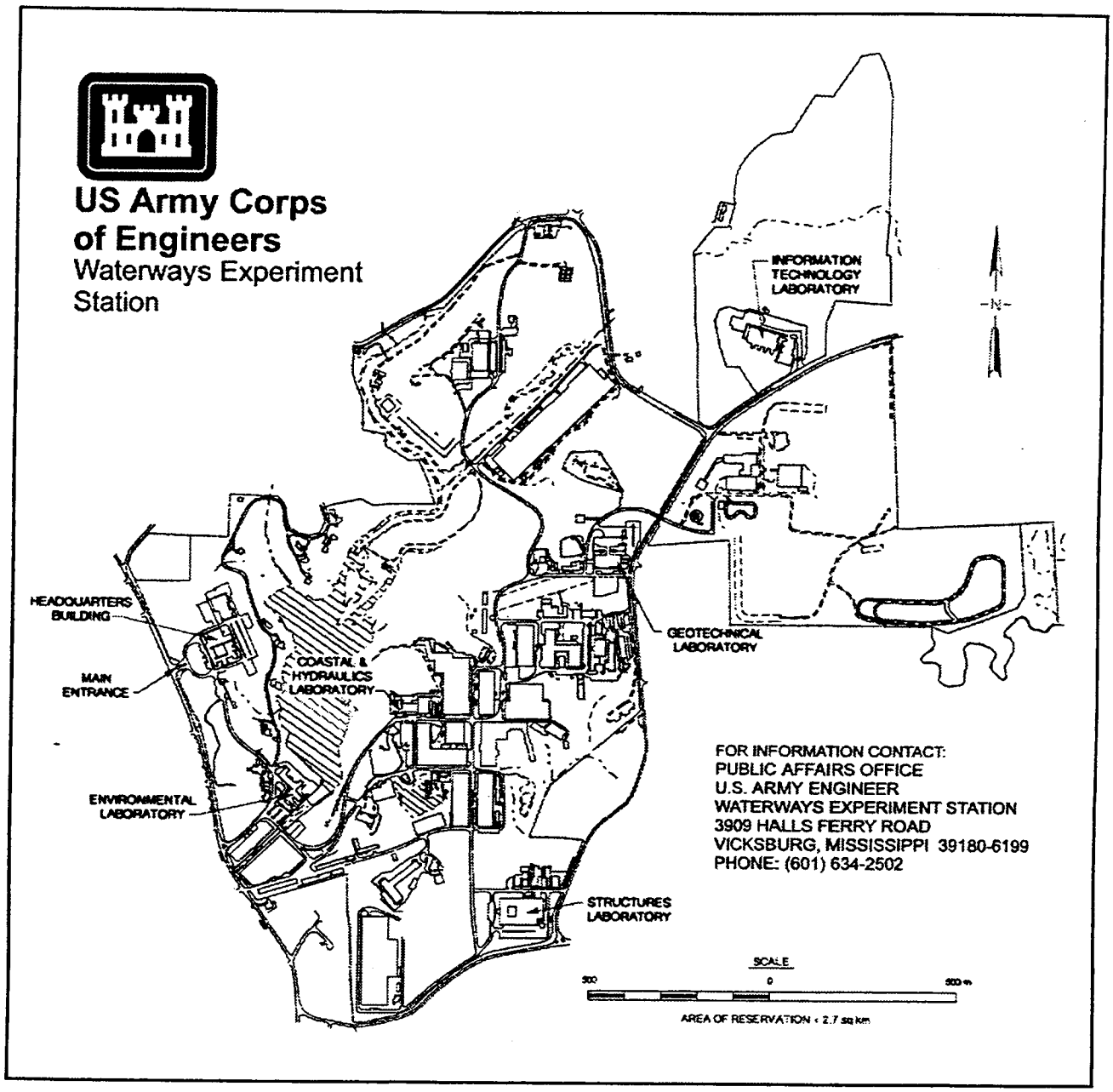

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Contents

Preface

The work reported herein was conducted as part of the Aquatic Plant Control Research Program (APCRP), Work Unit 32200 and Work Unit 32202. The APCRP is sponsored by the Headquarters, U.S. Army Corps of Engineers (HQUSACE), and is assigned to the U.S. Army Engineer Waterways Experiment Station (WES) under the purview of the Environmental Laboratory (EL). Funding was provided under Department of the Army Appropriation No. 96X3122, Construction General. The APCRP is managed under the Center for Aquatic Plant Research and Technology (CAPRT), Dr. John W. Barko, Director. Mr. Robert C. Gunkel was Assistant Director for the CAPRT. Program Monitor during this study was Ms. Denise White, HQUSACE.

This report was prepared by Dr. Judy F. Shearer, Aquatic Ecology Branch (AEB), Ecological Research Division (ERD), EL, WES. Technical assistance at WES was provided by Ms. Janis Lanier and Mr. Brian Durham. Field assistance was provided by personnel at the Lewisville Aquatic Ecosystem Research Facility, Lewisville, TX.

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1 Introduction and Objectives

Plant pathogen biocontrol research exploring the use of endemic microorganisms for management of noxious plant species had its beginnings in the United States over 30 years ago. By the early 1980's, an estimated 54 fungal taxa had been studied for their potential as mycoherbicidal agents on a variety of noxious plants (Templeton 1982). By 1991, the number had almost doubled (Charudattan 1991). The release of two mycoherbicides, DeVine and Collego, in the 1980's for management of strangler vine and northern joint vetch, respectively, demonstrated that fungi could be developed for commercial use.

The development of an endemic microorganism into a successful biocontrol agent involves several steps, including initiation, surveying, screening, risk assessment, formulation, release approval, field testing, and release or distribution (Wapshere, Delfosse, and Cullen 1989). Some steps are simple and straightforward; others can be time consuming and challenging. Not all steps are necessary; and some steps are dependent on the pathogen and its intended use as an augmentative or inundative agent.

During the initiation phase, information is gathered on the target plant and its associated diseases. If pathogens have been identified, they may be available from a source such as the American Type Culture Collection, and the survey step will be unnecessary or limited in scope. In most cases, and particularly with recent plant introductions, information is scant, pathogens are not available, and extensive survey work for disease-producing organisms becomes obligatory.

Included in the survey phase are collection, isolation, identification, and preservation of all potential pathogens. Coincident with field collecting, valuable ecological information on the host and pathogen can be gathered as they are examined in situ. The time frame for the survey phase may range from a few weeks to several years. Some survey work should remain a part of any endemic program because new pathogenic strains will, in all likelihood, develop as host and pathogen coevolve, and these new strains might have characteristics that could enhance the performance of an agent.

During screening, pathogens are evaluated for disease-producing potential on the target plant. Preliminary testing usually begins on a small scale in the laboratory by applying inoculum to excised plant parts, making visual assessment of

disease development, and assigning a damage rating. Preliminary results are confirmed by retesting those isolates that induce disease in the host. Only isolates that cause extensive damage to a host, i.e., have a high damage rating, are considered for further screening using rooted plants rather than leaf and/or stem segments. With larger scale experimentation, agent performance can be evaluated objectively by measuring differences in biomass between treated and control plants rather than by a subjective disease rating system. Optimum dose rate/disease development patterns and specific life cycle stages during which the host is most susceptible to ingress by the pathogen are determined during the screening step.

Because successful performance of a potential biocontrol agent in the laboratory cannot automatically be equated with successful performance in the field, small field plot testing during the screening step is a mandatory component of agent evaluation. Successful results during this stage are in part dependent on an understanding of host and pathogen ecology. Confirmation of laboratory findings relating to dose rates, seasonality effects, and life cycle stages can be assessed under natural conditions in the field.

Risk assessment encompasses host specificity testing and determining ecological adaptations of the pathogen in the field, including reproduction, dispersal, and survivability. Plants selected for host specificity testing include a range of closely related species, agronomically important plants, native species occupying similar habitats as the target plant, and endangered species. Rooted nonhost plants at different developmental stages, from seedlings to mature plants, are challenged with low- to high-dose rates of the pathogen, and the disease severity is recorded.

While endemic pathogens might be used to augment natural populations in the field (an augmentative approach), it is more likely that they will be developed and used in an inundative approach. The inundative strategy is to apply the pathogen to the target plant at levels that overcome plant defenses, thereby initiating an epidemic and bringing a plant population under manageable levels. With the inundative approach, the pathogen is used much like a herbicide. As such, it must be formulated into a product that has a shelf life and can be applied with conventional spraying equipment. During the formulation process, ways of producing mass quantities of the pathogen, either by wet or dry fermentation, must be researched. Optimally, the final product of the fermentation process should be spores or sclerotia because it is these life stages, rather than mycelium, that can withstand the necessary drying stage prior to incorporation into a carrier. The formulation step may be extremely time consuming because each biological agent has unique characteristics that must be dealt with on a case-by-case basis. There is no one universal formula that fits them all.

Regulations governing the release of endemic pathogens in the United States are dictated by their intended use as augmentative or inundative agents. Pathogens that are formulated for use as inundative biocontrol agents are regulated and registered under the Environmental Protection Agency (EPA) biorational pesticide guidelines (Charudattan 1991). If the pathogen is not endemic to the region in which the application is to be made, interstate movement becomes an issue, and

approval from both the United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS) and cooperating state agencies may be required. Pathogens that are used in an augmentative approach (i.e., supplementing a natural population) may not require a release permit if they are used in the state in which the pathogen was originally collected. If interstate movement is a factor, USDA and State agency permitting will most likely be required.

Once a formulated pathogen is registered with the EPA and required permits are obtained from Federal and State agencies, field testing and monitoring can be conducted under strict guidelines set up as part of an Environmental Use Permit. If the product is effective and deemed safe as a result of field testing, release and distribution can then begin. A pathogen developed as an inundative agent would be marketed and used in a manner similar to a herbicide. Successful use of a living biological product may be stringently dictated by the ecology of the host and pathogen. For example, if a pathogen is infective only during a host's seedling stage, its use may be limited to the beginning of a growing season.

In the 1970's, the U.S. Army Engineer Waterways Experiment Station (WES) began supporting research on the use of endemic plant pathogens for management of aquatic plants (Conway et al. 1979). One of the first agents examined was a fungal pathogen, *Cercospora rodmanii* Conway, as a potential biological control agent of waterhyacinth, *Eichhornia crassipes* (Conway et al. 1979; Freeman et al. 1981; Theriot, Theriot, and Sanders 1981; Pennington and Theriot 1983; Charudattan 1991). Abbott Laboratories, Inc. (Chicago, IL), developed a formulated product that was field tested at various localities in the southeastern United States. Poor field trial results suggested that the formulated agent was not efficacious when applied under conditions that favored rapid growth of the host (Charudattan 1991). In 1984, Abbott Laboratories decided not to register the product, and WES research on the use of the pathogen for waterhyacinth management was discontinued.

Over the next few years, endemic pathogen research was initiated on two submersed aquatic plants, *Hydrilla verticillata* (L.f.) Royle (hydrilla) and *Myriophyllum spicatum* L. (Eurasian watermilfoil). While results have been very promising using a strain of the endemic fungus *Mycoleptodiscus terrestris* (Gerd.) Ostazeski from Texas on hydrilla (Joye 1990, Joye and Cofrancesco 1991, Shearer 1996b), mixed results have been achieved with a strain of *M. terrestris* from Massachusetts on milfoil (Gunner 1983, 1985, 1987; Gunner et al. 1990; Shearer 1996a).

Current research efforts in the development of endemic fungal pathogens for management of the submersed aquatic plants hydrilla and Eurasian watermilfoil have focused on aspects of screening and formulation. The following chapters summarize pathogen research in four areas; formulation development, effect of seasonality on host/pathogen disease development, dose rate/disease development, and evaluation of fungal isolates for pathogenicity on milfoil.

2 Formulation Development

Introduction

Most formulation research on fungal biocontrol pathogens has been directed toward development of mycoherbicides to control terrestrial weeds. One of the primary considerations in this arena has been the retention of a wetting or dew period to prevent desiccation of the fungus until infection can be established within the host tissues (Hasan and Ayres 1990). While retention of fungal viability prior to application is a consideration in the development of a mycoherbicide for use on submersed aquatics, dew period in an aquatic system is not of major concern. For ease of application, the potential biocontrol agent is often encapsulated in a granule or pellet (Hasan and Ayres 1990). While formulations that are spherical in shape may work well in terrestrial habitats where they are incorporated into soil or broadcast for pre- or post-emergence weed control, their use in an aquatic system is severely limited because moving water easily dislodges them from the target plant and carries them away from the site of application.

Formulation development encompasses several steps, including manipulation of the fungus, design of carriers, and incorporation of the fungus into the carrier. Prerequisite to researching carrier prototypes, the compatibility of the fungus with various formulation inerts must be determined. The fungal component is further manipulated through processes of fermentation, milling, and drying to maximize colony forming unit (cfu) counts while maintaining viability and virulence of the pathogen.

Prototype formulations of *M. terrestris* were researched by Ricerca, Inc. (Painesville, OH). The classes of formulations evaluated for use in aquatic systems included spray-dried, invert emulsion, oil flowable, extruded granule, suspension concentrate, and fiber. A detailed report of Ricerca's formulation research efforts is included as Appendix A.

Materials and Methods

Hydrilla plants growing in clear acrylic columns (76 by 13.7 cm) were used for formulation evaluation. Plastic cups (1 ℓ) were filled to within 5 cm of the top with Brown's Lake sediment collected at WES and enriched with ammonium chloride (0.5 g/ℓ) and Esmigran (1.75 g/ℓ). The sediment was overlain with silica sand to inhibit algal growth. Plant material was collected from hydrilla culture tanks maintained in the biomanagement greenhouse. Three 15-cm apical segments of hydrilla were planted in each cup, and the cup was placed in the bottom of a column containing 12ℓ of Smart and Barko's (1985) nutrient solution. Air was gently bubbled into each column to provide water circulation.

Hydrilla formed a surface canopy in approximately 40 days. Four prototype formulations, an extruded granule, a suspension concentrate, and two invert emulsions were provided by Ricerca for evaluation (Table 1). The dry formulation (granules) was dispensed in 10-g aliquots to the surface of the water. The liquid formulations were dispensed in 20-ml aliquots. The suspension concentrate was poured onto the water surface, while the two invert emulsions were injected in a subsurface manner.

Table 1
***Mycoleptodiscus terrestris* Prototype Formulations Developed for Use in Aquatic Systems**

Formulation	Description
Granule	Wet pack, 35%; cellulose gum 7M, 10%; SeaSpen PF, 20% Celite 266, 8.7%; Aerosol R972, 6%; Nadex 360, 8.7%; and glycerin, 11.6%
Suspension concentrate	Light Karo syrup, 91%; wet pack, 4.5%; Celite 266, 3.1%; and Aerosol R972, 1.4%
Invert emulsion 1	Methyl oleate, 65%; wet pack 20%; Bentone 38, 5%; Witconol 14, 6%; and H_2O, 4%
Invert emulsion 2	Methyl oleate, 65%; wet pack, 20%; Bentone 38, 1%; Witconol 14, 6%; H_2O, 5%; and glycerin, 3%

The formulations were visually evaluated for their suitability of use in an aquatic system, adherence or plant coverage, and efficacy. Suitability of use was judged on ease of application and dispersal in an aqueous medium. Adherence or plant coverage was rated excellent, good, fair, or poor based on a visual estimation of plant surfaces coming into contact with the formulation as being 75-100, 50-75, 25-50, or 0-25 percent, respectively. Efficacy was subjectively evaluated by the presence or absence of characteristic disease symptoms.

Results and Discussion

The two invert emulsions and the extruded granule formulation most closely met the specifications for applicability in aquatic systems. The two inverts were viscous, and their application was somewhat problematic. Ideally, invert formulations work best when dispersed directly into the plant mat. Within the confining space of the small columns, application of the formulation in a subsurface manner was difficult. The extruded granules were lightweight, initially buoyant, and easy to apply. Within minutes after absorbing water they became swollen and spongy and began to slowly sink. As they drifted downward through the hydrilla mat, they became entangled in the vegetation.

The suspension concentrate was extremely thick and difficult to dispense. As the formulation came into contact with the water, it congealed into a ribbon and became well dispersed in the plant mat. Within minutes of dispersion, the water became cloudy. Although the water clears within a day or two, the appearance may make such a product undesirable for use in an aquatic system.

Coverage on the plant appeared to be related to the type of carrier and the location of application (i.e., surface or subsurface). Applied at the water surface, the granules could drift downward through the hydrilla mat. As they made contact with plant surfaces they became entangled on leaves and stems, resulting in excellent coverage of plant tissues. The suspension concentrate, when carefully poured over the water surface, congealed into thin ribbon-like strands that draped over plant surfaces as they wafted through the mat. Approximately 75 percent of the leaves and stems became covered with the ribbons. The viscous nature of the two inverts and the inability to apply them evenly throughout the water column diminished coverage on the plants to less than 50 percent. Plant mass in the lower half of the column received little inoculum. A greater volume of inoculum was delivered to plant parts in the upper half of the column, but it tended to clump, thereby lessening total contact area.

Even though the granules and the suspension concentrate dispersed evenly through the hydrilla mat and coverage was rated excellent, disease development did not ensue. Drying of the fungal mycelium following fermentation was a necessary prerequisite step to its incorporation into the carriers. Ricerca documented that drying the mycelium from 44 percent moisture to about 10 percent moisture resulted in a log reduction in cfu counts and in the ability of the fungus to rapidly grow on selective media (Appendix A). Direct plating of small aliquots of the granules and the concentrate used in the applications confirmed that fungal growth was retarded and cfu counts were well below the 1×10^6 range necessary for rapid disease development to ensue. The two invert emulsions were unevenly distributed through the plant mat, resulting in spotty coverage on plant tissues. Where the formulations came in contact with leaves and stems, the leaves and stems became chlorotic and flaccid, resulting in symptomatic disease expression following application with *M. terrestris* (Shearer 1996a). Subsurface sporulation of the fungus was evident, and sclerotia developed within the leaves.

Prototype work on *M. terrestris* formulations holds promise for development of a carrier that will be effective in an aquatic environment. While no single candidate met all the criteria for an ideal aquatic mycoherbicide, each of the tested prototypes had some characteristics that were highly desirable. For ease of use, a granular formulation would have the most widespread acceptance for professional application and for lay personnel. Extensive work on the fungal component will need to be forthcoming before any of the carrier prototypes can be effective. Methods of inducing the fungus to sporulate in fermentation culture must be explored. Not only would sporulation increase cfu counts, but it would reduce the problems associated with drying the mycelium. Induction of sclerotial formation might lead to a product with an extended shelf life. Without a viable and virulent pathogen, even the best delivery system would result in an ineffective product.

3 Host/Pathogen Responses to Seasonality

Introduction

Seasonal timing of pathogen application may be important in promoting disease development in a host plant population. While many diseases are induced to develop under specific environmental conditions, others are initiated at phenological points in a plant's growth cycle. For example, for an epidemic to ensue on a susceptible grass host, the fungus *Claviceps purpurea* (ergot of grasses) must invade the flower tissues within a short period following anthesis. With other diseases, plants may only be susceptible to invasion during the seedling stage, as leaves develop, or following pollination. After these periods in the developmental cycle, the plant becomes resistant to infection by the pathogen.

Phenological studies of Eurasian watermilfoil have suggested that control by chemical or mechanical means may be improved by timing implementation of management measures with plant phenological control points (Madsen 1993). With milfoil, these points are related to flowering and subsequent autofragmentation. In the southern United States, this event occurs during two periods of the growing season, midsummer and fall. If milfoil spread via autofragments is to be prevented, control measures need to commence before a surface canopy forms. While specific information on the phenological control points for hydrilla is currently under investigation, it is likely that during certain periods of its growth cycle, hydrilla may be more susceptible to herbicide injury or attack by fungal pathogens.

To determine if seasonality (i.e., plant phenology) is an important factor in hydrilla susceptibility to fungal attack, small plots within a hydrilla-planted pond were treated with the fungal pathogen *M. terrestris* at monthly intervals, June through August 1995. One month post-application, aboveground biomass was collected in treated and control plots to determine how effectively the pathogen impacted the host plant population.

Materials and Methods

Twenty-four plots were set up in a hydrilla-planted pond at the Lewisville Aquatic Ecosystem Research Facility, Lewisville, TX, in June 1995. The plots measured 1 by 1 m at the water surface and averaged approximately 1.2 m in depth. Because the inoculum was applied as a liquid, the plots were enclosed in plastic sheeting to reduce dissemination and dilution of the inoculum outside the plot boundary. At monthly intervals, starting in early June, 4 of the 24 plots were treated with a broth slurry of the fungus *M. terrestris*. One month post-inoculation, aboveground biomass was harvested to determine differences in plant dry weight between treated and control plots. Similar treatments and harvests followed in July and August.

The inoculum was prepared by starting seed cultures of *M. terrestris* on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) plates. The seed cultures were incubated in the dark at 28 °C for 7 days. Agar plugs were cut with a sterile #4 cork borer from around the leading edge of the colony. Five plugs of the fungus were added to 1-ℓ Erlenmeyer flasks, each containing 500 ml of modified Richard's V8 juice broth (glucose, 10 g; KNO_3, 10 g; $CaCO_3$, 3 g; V8 juice, 200 ml; H_2O, 800 ml). The flasks were placed on a platform shaker (New Brunswick, Edison, NJ) set at 200 rpm. After 6 days, the mycelial mat that developed in the flasks was filtered through 4 layers of cheesecloth and ground in a blender for 30 sec. Sterile water was added to the fungal slurry to give an inoculum concentration of approximately 1×10^6 cfu/ml. Aliquots of 2 ℓ were applied to the surface of treated plots at monthly intervals, June, July, and August.

Four weeks post-inoculation, aboveground biomass in treated and control plots was harvested by divers using scuba equipment. All biomass inside the plot boundaries was cut at the sediment surface, bagged, and labeled. A 10-g sub-sample of plant material from each plot was placed in a plastic bag and returned to the biocontrol laboratory at WES for microbial analysis. The remaining biomass was washed, placed in preweighed paper bags, dried in a plant drying oven set at 60 °C for a minimum of 5 days, and weighed to determine plant biomass dry weight.

Dilution plating was used to determine microbial counts and frequencies in hydrilla stem tissue. Each 10-g subsample of plant material was thoroughly washed, submerged in a 10 percent chlorox solution for 1 min to eliminate surface contaminants, and rinsed in sterile water for 3 min. The tissue was ground for 30 sec in a sterile blender containing 100 ml of water. Aliquots of the plant slurry were pipetted into sterile water blanks to give dilutions of 1/100 and 1/500. After thorough shaking, 1-ml aliquots of the dilutions were distributed over the surface of Martin's agar plates (3 plates per dilution). The plates were incubated in the dark at room temperature for 5 days. Total colony counts and the number of *M. terrestris* colonies in each sample were determined by visual examination.

Results and Discussion

Aboveground biomass of hydrilla was reduced following treatment with the fungus *M. terrestris* at each treatment period, June, July, and August (Figure 1). Better control was realized earlier in the growing season. Aboveground biomass was reduced 75 and 63 percent in June and July, respectively, compared with the controls, but only 33 percent in August. At the time of the first application, hydrilla was actively growing but had not yet reached the surface of the water. The height of the mat inside the plot boundaries was approximately 17 cm below the surface of the water.

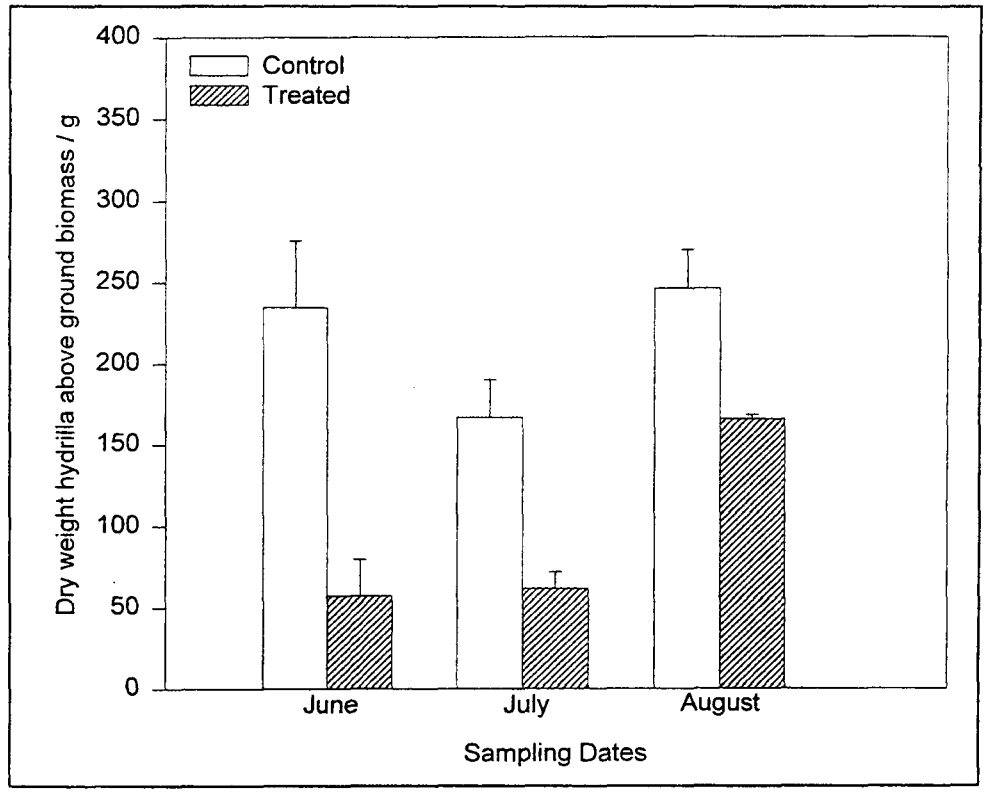


Figure 1. Dry weight hydrilla aboveground biomass collected June, July, and August 1995 from treated and control plots 1 month post-inoculation with *M. terrestris* (Bars indicate ± SE)

Young plant tissues may be more susceptible to ingress by *M. terrestris* than are older plant tissues. The fungus is thought to secrete enzymes, cellulases, and pectinases, whereby it can enter directly through the cell wall (Joye and Paul 1992). Well-developed, mature plant tissues may present resistance to mechanical pressure and to enzymatic activity by means of their increased thickness and their modified structure (Agrios 1969). Changes in phytoplane surface deposits during a growing season may also affect the ability of a pathogen to gain ingress into a

host. As the season progresses, there is a buildup of microorganisms, epiphytic algae, and inorganic mineral deposits on the plant surfaces. Such buildup may present a physical barrier to fungal entry, or it may interfere with physiological processes necessary for fungal recognition of a host.

Temperature may also be a major factor in disease development. Cooler temperatures earlier in the growing season are much more favorable to fungal growth than are elevated temperatures which occur in the ponds at the Lewisville facility later in the growing season. The optimum temperature for fungal growth is between 22 and 28 °C, which approaches the high temperature reading early in the season. Later in the growing season, the pond temperatures easily exceed 30 °C both at the surface and at middepth, a temperature which affects growth and virulence of the fungus.

Samples of hydrilla tissue harvested from the experimental pond prior to the first fungal application indicated that *M. terrestris* was not present in the pond as a naturally occurring endemic organism. The fungus does not appear to be long-lived in the aquatic environment nor to become established as an endophyte in hydrilla tissue. This is indicated because 1 month after inoculation, June, July and August, *M. terrestris* was not isolated from treated plots (Table 2). The cfu counts from hydrilla plant tissue were fairly consistent from treated and control plots for each collecting period except August. At the time of the final biomass harvest in August 1995, algal buildup covered over 50 percent of the water surface. Elevated temperatures and anaerobic conditions in some areas of the pond may have contributed to the decline from the previous months. The slightly higher cfu counts in July were attributable to an increase in the number of isolated yeasts.

Table 2
Average Number of Fungal cfu/g of Hydrilla Plant Tissue Collected Pre-Inoculation and 1 Month Post-Inoculation with *M. terrestris* from Treated and Control Plots, Summer 1995

Treatment Period	Treated		Control	
	Total[1]	*M. Terrestris*	Total[1]	*M. Terrestris*
Pre-inoculation	NA	NA	24.45	0
June	6.67	0	29.17	0
July	6.25	0	50.00	0
August	6.33	0	00.92	0

[1] Number of cfu/g hydrilla plant tissue.

4 Host/Pathogen Dosage Response

Introduction

Plant pathogens are used in a number of ways as biological control agents. In a classical approach, exotic or endemic pathogens are released and allowed to spread naturally through a host population. Disease development becomes a function of natural constraints on the pathogen and the host (TeBeest 1993). Reintroductions should be unnecessary because the pathogen survives from year to year, providing long-term control of the target plant. In the United States, one of the best examples of the use of a classical strategy was the introduction of the fungus *Entyloma compositarum* into Hawaii for management of hamakua pamakani, a weed of rangeland.

By contrast, the inundative approach, often called the mycoherbicide strategy, uses endemic plant pathogens as management tools in ways consistent with chemical herbicides (TeBeest 1993). A pathogen is applied in volumes and dosages to a targeted plant population to achieve desired control in a timely and effective manner. The manipulation of inoculum, according to Holcomb (1982), is the only constraint against disease development that can be controlled by man; little can be done to alter the host, climate, temporal, or spatial features of the pathosystem. It is the lack of sufficient inoculum which most likely limits disease epidemics in nature (Shrum 1982). Predicting the correct amount of inoculum and its timely application may mean the difference in success and failure of the pathogenic agent.

M. terrestris, an endemic pathogen used in an inundative manner, has been shown to significantly reduce aboveground biomass of hydrilla within 2 weeks following application with a mycelial slurry of the fungus (Joye 1990, Shearer 1996b). Disease symptoms first appear as interveinal chlorosis on hydrilla leaves within 5 days post-inoculation. As the fungus ramifies in the plant, leaves become flaccid and disintegrate, followed by stem collapse and disarticulation. After 2 weeks, the epidemic wanes, incidence of infection lessens, and plants that are not killed are capable of regrowth. These events have been documented in a previous dosage response experiment (Shearer 1996b). Hydrilla grown in 2,200-ℓ tanks was treated with low, medium, and high levels of a broth slurry of *M. terrestris*

fungal inoculum rated at 1 x 10^5 cfu/ml. At 2 weeks post-inoculation, percent cover of hydrilla at the surface of the tanks was estimated to be 91, 75, 55, and 25 percent for control, low, medium, and high dose treatments, respectively. Plants treated with medium and high doses of *M. terrestris* were largely defoliated, and only bare stems remained rooted in the sediment. At sites on the plant that did not come in contact with fungal inoculum, green tissue was evident. As the effect of the application lessened, hydrilla began to regrow.

A severely damaged hydrilla population, weakened by disease induced by a single medium dose of fungal inoculum, may not be able to regrow if subjected to a second dose of the fungus when the initial epidemic is beginning to subside. The effectiveness of repeating an application of a fungal pathogen on a host plant population was tested on hydrilla grown in 2,200-ℓ tanks. Hydrilla plants received one medium dose of the fungus *M. terrestris* followed 2 weeks later by a second and equal dose of the same organism.

Materials and Methods

Tanks, 183-cm diam by 70-cm deep (approximately 2,200 ℓ), were planted with hydrilla. Plastic containers 36 by 30 by 13 cm were filled with sediment to a depth of 8 cm and overlain with 4 cm of washed silica sand. Each container was planted with 30 apical tips of hydrilla 15 cm in length . Twelve containers were placed in each of seven tanks, and the tanks were then filled with Smart and Barko's (1985) nutrient solution. The plants were allowed to grow until they formed a distinct canopy at the water surface before treatment was applied.

The inoculum was prepared for the field studies as described in Chapter 3. Treated tanks received 1 ℓ of inoculum which was distributed by pouring the fungal slurry over the water surface. Half the treated tanks received an additional 1 ℓ of inoculum 2 weeks following the first application. One month later, the total aboveground biomass was collected from each tank. The aboveground biomass was clipped at the sediment surface, washed to remove any sediment or sand, dried at 60 °C for 5 days, and weighed.

Results and Discussion

Following application of the fungal pathogen *M. terrestris*, disease symptoms similar to those previously reported (Joye 1990, Shearer 1996a) developed on hydrilla. Within 1 week post-application, plants became chlorotic and defoliated. Within 2 weeks, extensive fragmentation resulted in pieces of hydrilla stem tissue floating on the surface of the water in the treated tanks. By 1 month post-application, there was a significant reduction in hydrilla aboveground biomass of treated tanks compared with untreated controls (Figure 2). The second application of *M. terrestris* produced only an additional 5 percent reduction in biomass compared with tanks receiving a single application of the fungus.

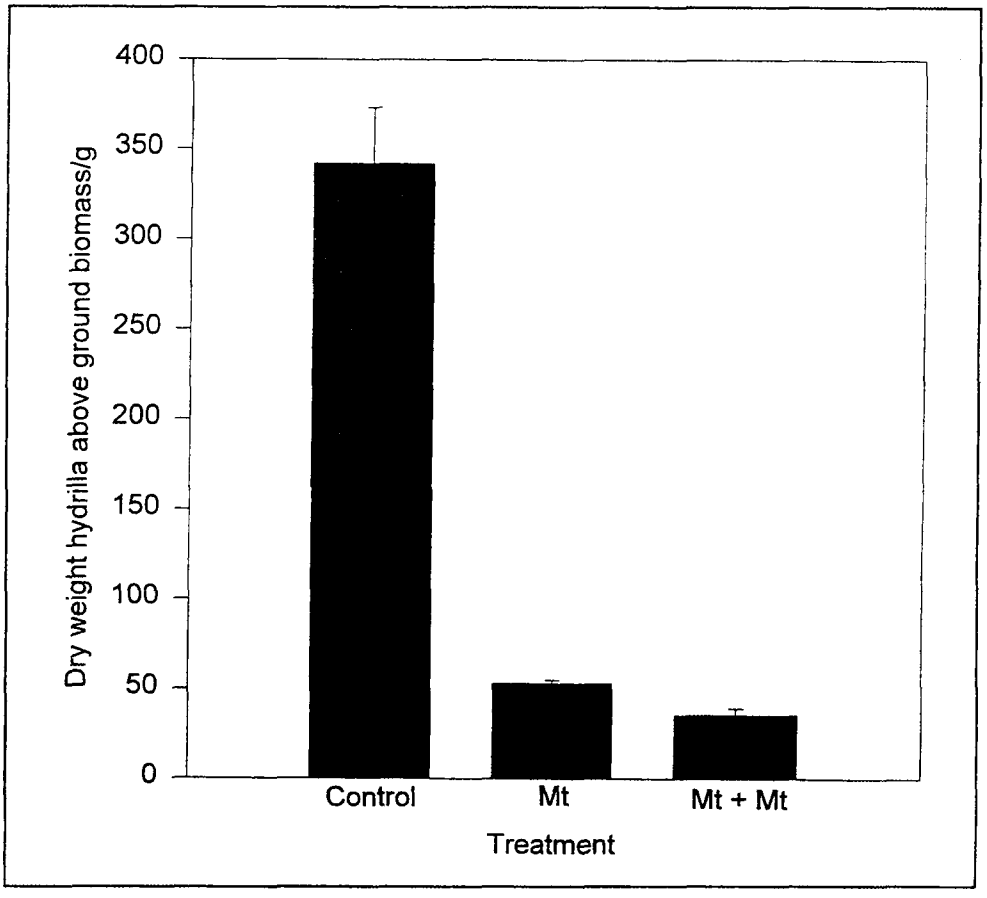


Figure 2. Dry weight hydrilla aboveground biomass collected from treated and control tanks 1 month post-inoculation with *M. terrestris* (Mt = one fungal treatment; Mt + Mt = two fungal treatments) (Bars indicate ± 1 SE)

The ineffectiveness of the second application of *M. terrestris* may be attributable to several factors. One is related to the small amount of biomass remaining in the tanks following the first application. *M. terrestris* requires contact with the host before attachment and disease development can ensue. In a thick plant mat, chances of contact with the host are great, as the inoculum is dissipated in the water. When the mat is considerably reduced, resulting in widely spaced plant stems, most of the inoculum would settle to the bottom of the tank without coming into contact with plant tissue.

Another possibility is that plant defense mechanisms activated in response to the first application of the fungal pathogen may have been effective in warding off infection following the second application of the fungus. Research with certain pathogens has shown that susceptible plants may become resistant to a pathogen if they are inoculated with a nonlethal dose of the pathogen during the seedling stage. This event seems unlikely with hydrilla because the plants were mature at the time of application, and the amount of the dose should have overcome any ability of the plant to resist attack by the pathogen.

Although the possibility exists, it is unlikely that the fungus was avirulent or lacked viability. The isolate used in the applications is one that has produced consistent results in laboratory, greenhouse, and tank studies when applied at a concentration of 1 x 10^6 cfu/ml. Viability of the fungus was confirmed when cultures readily grew from dilutions of the fungal slurry used to estimate cfu rates at application time.

5 Milfoil Pathogen Screening

Introduction

The fungus *M. terrestris* was researched as a potential mycoherbicide for management of Eurasian watermilfoil by EcoScience, Inc.,Worchester, MA (Gunner 1983, 1985, 1987; Gunner et al. 1990). A formulation was developed whereby the fungus was grown in broth culture in industrial fermentors and incorporated into a calcium alginate matrix. For field application, the mycoherbicide was extruded as pellets or strings. Both designs were tested on a small scale in a milfoil-planted pond at the Lewisville Aquatic Ecosystem Research Facility at Lewisville, TX, in 1992. The following year, an efficacy test was performed on field plots set up on a milfoil-infested pond adjacent to Guntersville Reservoir (Shearer 1996b). Following poor field test results, the company decided not to invest further efforts toward development of *M. terrestris* as a mycoherbicide. Three major research efforts would have been required for redesign of the mycoherbicide; the formulation needed major modifications to make it more effective in an aquatic environment, the biocontrol fungus agent needed to be reevaluated for disease-producing potential on milfoil, and improvements were required in laboratory handling of the fungus in terms of fermentation, milling, and drying.

In an effort to determine if the Massachusetts isolate of *M. terrestris* was still viable and virulent, it was reevaluated in the biocontrol laboratory and greenhouse facilities at WES. At the same time, additional *M. terrestris* isolates, collected from milfoil tissue from different geographic regions within continental United States, were screened (Table 3). From among the isolates that showed positive results in the screening process, further greenhouse and field evaluations were conducted to compare their performance with the Massachusetts isolate.

Table 3
Results of Screening *M. terrestris* Isolates from Different Geographical Regions of the United States on Eurasian Watermilfoil Planted in 50-ℓ Aquaria

Isolate Identification	State	Biomass Reduction, %
25A	Texas	67
EcoMt	Massachusetts	88
70293	Alabama	27
59293	Washington	85
19593	California	88
87093	Vermont	56

Materials and Methods

Screening

Apical stem pieces of milfoil were collected, washed, cut in 15-cm lengths, and planted in sediment-filled plastic inserts measuring 2 by 2 by 2 in. (3 tips/insert). The sediment surface was overlain with silica sand to inhibit algal growth. The inserts were placed in wide-mouthed 1-ℓ glass jars containing a nutrient solution (Smart and Barko 1985), and the plants were allowed to grow for 2 weeks in a 25 °C growth chamber set on a 14-hr photoperiod.

Aquarium studies

Isolates that produced a greater than 75 percent reduction in aboveground biomass of milfoil in the small-scale screening were considered for testing on a larger scale in 50-ℓ aquaria. Time constraints limited the number of isolates which could undergo further testing prior to the field season. Higher priorities were assigned isolates from different geographical regions. Approximate 1-ℓ plastic cups were filled with sediment to within 5 cm of the top of the cup and overlain with silica sand. Three 15-cm apical sprigs of milfoil were planted in each cup. Four cups were placed in an aquarium and covered with nutrient solution (Smart and Barko 1985). After the milfoil plants reached the surface of the water (approximately 1 month), fungal inoculum was applied.

Field studies

Based on greenhouse performance, two isolates, 19593 and 59293, from California and Washington, respectively, were selected for field testing in addition to the Massachusetts isolate of *M. terrestris*. One-meter field plots, as described

previously for the *M. terrestris*/hydrilla study (Chapter 3), were constructed on a milfoil-planted pond at the Lewisville Aquatic Ecosystem Research Facility at Lewisville, TX.

Inoculation and harvesting

During the study, *M. terrestris* isolates were temporarily maintained on agar slants in a refrigeration unit at 4 °C. They were retrieved from temporary storage as needed, and a fungal broth slurry was prepared as described in Chapter 3. Inoculum was applied at rates of 1, 20, and 2,000 ml to the 1-ℓ jars, 50-ℓ aquaria, and 1-m field plots, respectively. The inoculum was distributed over the water surface and was brought into contact with plant surfaces through natural dissipation in the water column. Aboveground biomass was collected at 2 weeks and at 1 month post-inoculation for greenhouse and field studies, respectively. The plant material was cut at the soil surface, placed in preweighed paper bags, dried at 60 °C for 5 days, and weighed for biomass dry weight determinations. Effectiveness of the treatment was calculated by determining the percent reduction in aboveground biomass of treated plants compared with untreated controls. Treatments were replicated five times for greenhouse studies and three times for field studies.

Results and Discussion

A total of 30 *M. terrestris* isolates from different geographic regions within the United States were screened in a small jar bioassay for pathogenicity on milfoil (Table 4). Seventeen of the isolates reduced aboveground milfoil biomass greater than 75 percent compared with uninoculated controls and were considered for additional testing on milfoil grown in 50-ℓ aquaria. Five isolates, 69493, 70393, 114493, 73992, and 59292, performed well in the small scale screen test but failed to grow when plated onto PDA as seed cultures for aquarium testing. Time and equipment constraints further limited the number of remaining isolates that could be tested on a larger scale prior to the beginning of the 1994 field season. With a limited number of aquaria and each test run requiring a minimum of 2 months, not all isolates could undergo further testing in aquaria in the 5-month time period before field evaluation had to begin. Therefore, isolates from different geographical regions were given priority. Based on performance in aquarium studies (Table 3), isolates from Massachusetts, Washington, and California were selected for field evaluation. The Massachusetts isolate (EcoMt) had been used previously by EcoScience as a potential biocontrol for milfoil (Stack 1990).

From each batch of inoculum prepared for field application of the *M. terrestris* isolates EcoMt, 19593, and 59293, a small quantity was reserved for a concurrent greenhouse aquarium study to test viability and virulence of the three isolates. After 2 weeks, no biomass remained in the aquaria treated with isolate 19593, and biomass was reduced 95 and 99 percent with isolate 59293 and EcoMt,

Table 4
Isolates of *M. terrestris* Screened for Pathogenicity on Eurasian Watermilfoil

Isolate	State	Biomass Reduction, %	Disposition[1]
25A	Texas	76	Phase II screening*
59293	Washington	76	Phase II screening*
EcoMt	Massachusetts	76	Phase II screening*
FHY18	Texas	75	discarded
HSR25D	Texas	64	discarded
FLMt	Florida	37	discarded
64493	Alabama	55	discarded
64793	Alabama	68	discarded
62593	Alabama	51	discarded
65893	Alabama	71	discarded
66893	Alabama	61	discarded
69493	Alabama	79	no grow
68793	Alabama	61	discarded
19593	California	79	Phase II screening*
19793	California	80	Phase II screening
70393	Alabama	85	no grow
59193	Alabama	73	discarded
114493	Texas	90	no grow
69993	Alabama	88	Phase II screening
72293	Alabama	88	Phase II screening
70293	Alabama	88	Phase II screening*
69693	Alabama	72	discarded
WIMt	Wisconsin	65	discarded
5392	Alabama	76	discarded
73992	Alabama	79	no grow
34692	Tennessee	79	Phase II screening
59292	Alabama	76	no grow

(Continued)

[1] Phase II screening = Isolates that produced greater than 75 percent reduction in above-ground biomass were considered for further testing; * = Isolates that underwent additional screening in 50-ℓ aquarium studies; no grow = Isolates that did not retain viability.

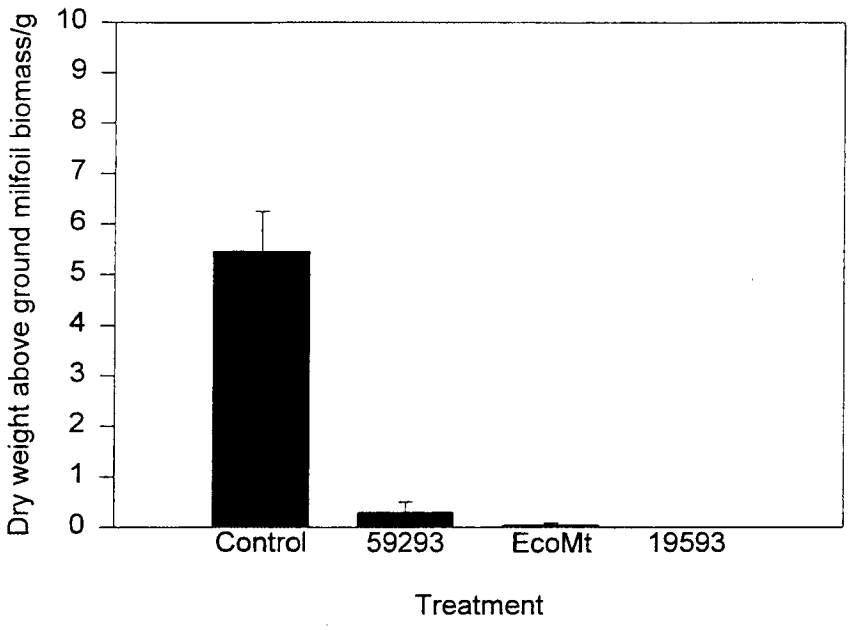
Table 4 (Concluded)			
Isolate	State	Biomass Reduction, %	Disposition[1]
78592	Alabama	72	discarded
87092	Vermont	85	Phase II screening*
87593	Vermont	77	Phase II screening

respectively (Figure 3a). Similar results were not obtained from the field study. Only slight reductions in biomass were found in treated plots compared with untreated controls (Figure 3b).

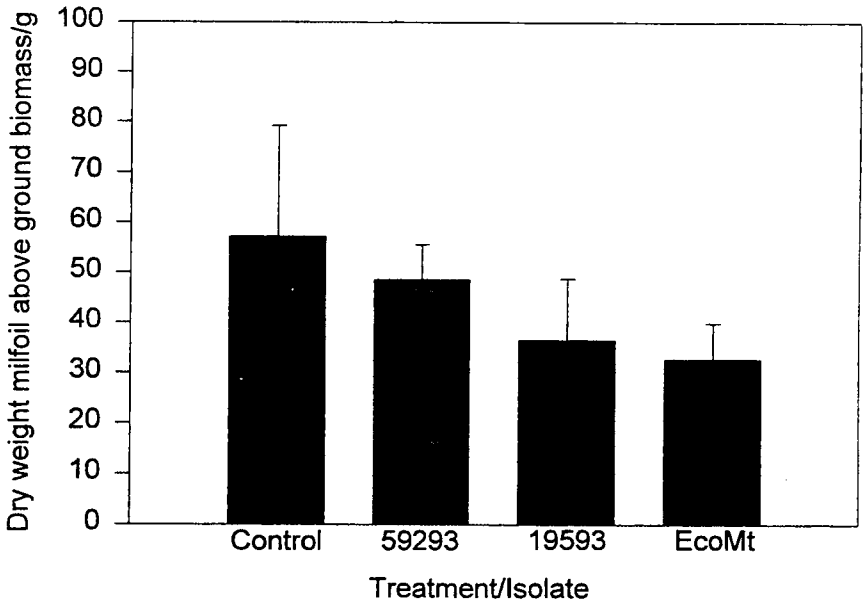
The substantial differences in results between the concurrent greenhouse and field study cannot be attributed to the inoculum because the same batches were used for both studies. Effective cfu counts of the treatments (taking into account the dilution factor of adding a liquid inoculum to an aqueous medium) were slightly less for the greenhouse study than for the field application. In effect, less inoculum per volume produced better results on greenhouse treated plants.

Greenhouse and field grown milfoil plants appeared quite different macroscopically at the time of inoculation. Greenhouse plants were intact and had bright green leaves and stems free of algae and other inorganic deposits on their surfaces. In contrast, plants in the ponds had begun autofragmenting. Leaves were beginning to brown, and plant surfaces were encrusted with algae and inorganic deposits. Such coverage may provide a physical barrier to invasion by the pathogen since the pathogen may not be able to grow through the encrustation to reach the plant surfaces. Lack of direct contact with host tissues may also inhibit fungal development because certain recognition factors stimulatory to growth and invasion of the pathogen would not be initiated.

Poor field performance may also be a factor of timing and site of application. Milfoil is more commonly found in cool, temperate regions of the United States rather than warmer, more southerly regions. Daily ambient temperature maximums of 38 °C were not uncommon throughout the duration of the study. Under such conditions, water temperatures at the surface and at middepth in the shallow ponds can easily exceed 32 °C in July and August. While the fungal pathogen will grow at high temperatures, virulence seems reduced when temperatures exceed 30 °C (Shearer 1995). Due to the high temperatures, milfoil plants were undergoing senescence and fragmentation. In the plant life cycle, inoculum might be more effective on milfoil if applied before the onset of senescence. Application early in the growing season, April or May, would avoid both temperature extremes in the ponds and the buildup of encrustations on the plant surfaces.



a. Dry weight milfoil aboveground biomass collected from 5-ℓ aquaria 2 weeks post-inoculation with strains of *M. terrestris*, 59293, EcoMt, and 19593 (Bars indicate ± 1 SE)



b. Dry weight milfoil aboveground biomass collected from treated and control plots 1 month post-inoculation with strains of *M. terrestris*, 59293, 19593, and EcoMt (Bars indicate ± 1 SE)

Figure 3. Dry weight milfoil aboveground biomass following inoculation with the fungal pathogen *M. terrestris*

6 Conclusions and Recommendations

Prototype work on *M. terrestris* formulations holds promise for development of a carrier that will be effective in an aquatic environment. One of the main criteria for formulation success, the ability to adhere to hydrilla under submersed conditions, was met by each of the formulations tested, invert emulsions, granules, and the suspension concentrate. For ease of application, the granular formulation was superior to both the suspension concentrate and the inverts. Maintaining fungal viability through formulation processes of fermentation, milling, and drying has been, and continues to be, a formidable problem that must be overcome before a marketable product can become a reality. Laboratory experimentation with the fungal component must include investigation of methods that will induce the pathogen to sporulate during wet or dry fermentation. Incorporating spores rather than mycelia in a formulation matrix can significantly increase viability and cfu counts. At present, cfu counts using a mycelial matrix rarely exceed 1×10^6 cfu/ml, whereas counts greater than 1×10^8 cfu/ml, can be realized through induction of sporulation. It is of primary importance that high cfu counts can lessen the volume of inoculum required to initiate a disease epidemic in a host plant population.

Seasonality may play a role in the susceptibility of hydrilla and milfoil to ingress by a fungal pathogen. Timing inoculum application with certain potential control points or "weak spots" as described by Madsen (1993) could result in better control. Preliminary results indicate that hydrilla is more susceptible to pathogen ingress early in the growing season, a period during which temperatures are more conducive to optimum fungal growth and development. Additional studies with both hydrilla and milfoil under field situations will be necessary to evaluate phenology of the host with disease development by a pathogenic organism.

M. terrestris fungal inoculum applied at a medium-dosage rate produces a substantial reduction in aboveground biomass of hydrilla within 2 weeks post application (73 percent compared with untreated controls). A second application of inoculum was shown to yield only an additional 5 percent reduction to the standing biomass of aboveground hydrilla tissues. Any hydrilla tissue that does

not succumb to disease is capable of regrowth, and a reinfestation could develop within a relatively short period of time. Current research shows that an integrated approach, combining an application of the fungus with the herbicide fluridone, appears to hold the most promise for inhibiting regrowth of hydrilla (Netherland and Shearer 1996).

While excellent control of milfoil can be realized with applications of fungal pathogens in the laboratory and greenhouse, similar results have not yet been achieved under field situations. The discrepancies may be attributable to the pathogen, phenology of the plant, water temperature, or presence of algae and other deposits on plant surfaces. Further research on milfoil will address these areas. Due to time constraints prior to the 1994 field season, only a minimal number of potential milfoil pathogens were retrieved from the culture collection for pathogenicity screening. Continued studies will determine if more virulent agents can be identified that can be used alone, in association with other biocontrol agents, or in an integrated approach with herbicides.

In the future, more emphasis must be placed on classical biological control using plant pathogens. The method has proven extremely successful with insect agents. That it works with plant pathogens has been demonstrated using the rust *Puccinia chondrillina* for management of skeleton weed in Australia and the white rust *Entyloma compositarum* for management of hamakua pamakani in Hawaii. Limited survey work has been undertaken in Europe for pathogens of milfoil and in China for pathogens of milfoil and hydrilla. Preliminary results from the investigations have indicated that a large complex of pathogenic microorganisms are found in association with hydrilla and milfoil in their native habitats. Expanded survey work throughout a greater portion of the broad geographical range of the two plant species may yield a useful array of potential biocontrol pathogenic agents.

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Appendix A
Formulation of the Fungus *Mycoleptodiscus terrestris* to Increase its Potential as a Mycoherbicide of the Aquatic Weed *Hydrilla*

The report "Formulation of the Fungus *Mycoleptodiscus terrestris* to Increase its Potential as a Mycoherbicide of the Aquatic Weed *Hydrilla*" was submitted upon completion of a Broad Agency Agreement contract between Ricerca, Inc., Painesville, Ohio, and the U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS. The research objective was to develop prototype formulations of the endemic fungus for use on the submersed aquatic plant *Hydrilla verticillata*. Included in the report are detailed descriptions of each aspect of formulation development, including fungal fermentation, milling and drying, inert evaluation, carrier development, and evaluation of selected formulations. Development of a marketable aquatic mycoherbicide is dependent on effectiveness of the two formulation components; the biological agent and the carrier. Carrier research was the primary focus of the agreement because one of the major hurdles in aquatic mycoherbicide development is finding a combination of inerts that would adhere to plants submersed in water.

Formulation of the Fungus *Mycoleptodiscus terrestris* to Increase its Potential as a Mycoherbicide of the Aquatic Weed *Hydrilla*

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Contents

Preface

This report was prepared to document services completed between July 1994 and January 1995 by Ricerca, Inc. under contract to the Aquatic Weed Lab, U.S. Army Engineer Waterways Experiment Station, Contract No. DACW39-94-C-0098. Project participants were: Formulations Section: Kevin E. Crosby (Section Head), William S. Hanson, Melvin J. Long, and Timothy J. Cardina; Biocontrol/Fermentation Section: Suzan H. Woodhead (Section Head and Principal Investigator), Edward W. Dixon, Jacqueline P. Soles, Susan C. Rabatin, Richard T. O'Donnell, and Daniel J. O'Leary. Report authors were Daniel J. O'Leary, Timothy J. Cardina, and William S. Hanson.

Summary

Experimental formulations of the fungus Mycoleptodiscus terrestris were prepared to improve its demonstrated efficacy as a mycoherbicide of the aquatic weed Hydrilla sp. under controlled conditions. Preliminary studies demonstrated compatibility of the fungus with various formulation inerts, and the degree of drying and milling which could be applied to the fungus without significant loss of viability. Four 20 L fermentations were completed and the harvested wet packs were used in trial formulations. The general classes of formulations prepared and evaluated were: spray-dried, invert emulsion, oil flowable, extruded granule, suspension concentrate and fiber. Invert emulsion and extruded granule formulations demonstrated properties closest to the desired. The extruded granules hydrated and took on a gel or spongy appearance. A portion of the hydrated granules remained at or below the water surface and a portion slowly descended to the bottom. The inverts floated on or just below the water's surface. It is believed that moderating the entrapment of air in the invert during mixing of the aqueous and oil phases at application would reduce the buoyancy of the formulation. An alternative would be to apply the invert below dense vegetation so that it floats up and becomes entangled in the weed. The fungal biomass produced in submerged fermentation consisted of mycelium with no specialized structures for survival. Because of this the viability and vigor of the formulated fungus declined in a matter of weeks at approximately 5°C. Future efforts should include strain selection, experiments to determine conditions which result in highly infective and stable units of the fungus, and optimization of fermentation and formulation.

Introduction

Mycoleptodiscus is a pathogen of Hydrilla and various other water weeds with potential to be developed into a mycoherbicide for use in waterways. The fungus is effective under controlled conditions, but not under field conditions. It is believed that the lack of efficacy in the field is due to failure of the fungus to contact the target long enough for infection to take place. A formulation which improves contact with the target is required. The first objective of this research was to develop a formulation incorporating a carrier which suspends the viable fungus in water for 24-36 h. This would allow passive interception of the fungus by the target weed. The second objective was to have the formulated organism adhere to Hydrilla, allowing sufficient contact time for infection. Both objectives are critical to the practical success of the mycoherbicide.

Preliminary Studies

Biomass of Mycoleptodiscus terrestris isolate A5 was produced in shake-flasks for use in experiments to determine compatibility with formulation inerts, effect of milling, and effect of drying on the fungus.

Shake-flask production

Three Fernbach flask shake cultures were each produced in 500 mL of modified Richard's medium (V8 based medium) on a shaker at 250 rpm and 25 C for 7 days. The biomass was collected on Miracloth™ (Calbiochem Corp.) supported in a buchner funnel. Yields were: Flask 1, 64.7 g; Flask 2, 91.8 g; and Flask 3, 75 g. Water made up about 93% of the weight of biomass from Flask 3. The fungus was observed microscopically and biomass was found to be mycelium. No spores were observed, though there were rounded hyphal cells that might be more resistant to drying/milling than the standard filamentous hyphal cells.

Compatibility of Mycoleptodiscus biomass with formulation inerts

Well-plates (24-well Cell Wells™, Corning) were used to determine in vitro compatibility of the fungus with thirty-six inerts. Wells containing a nutrient medium (1/4 strength potato dextrose broth, Difco Laboratories) and the fungus (biomass from Flask 3 above) were amended with 10, 100, and 1,000 ppm of each inert. Growth of the fungus was evaluated after one week of incubation at 25°C (Table 1).

Table 1
Inhibition of growth of mycelial inoculum of M. terrestris by formulation inerts

Inert	Growth inhibition[a]
1. Lattice NTC	NE
2. Seaspen PF	NE
3. Polargel HV	NE
4. Reax 83A	NE
5. Reax 85A	NE
6. Geropon T-77	Severe to complete at 1000 ppm
7. Barden Clay AG1	NE
8. Stepwet 95	Severe in two reps at 1000 ppm
9. Lomar D	NE
10. Stepwet DF90	Mod - severe, 2 reps at 1000 ppm
11. Stepsperse DF400	NE
12. Lomar PWA	NE
13. Avicel PH101	NE
14. Geropon TA72	NE
15. Alkamide DS-280/S	NE
16. Morwet D425	NE
17. Morwet EFW	NE
18. Volclay bentonite	NE
19. Kaolin clay	NE
20. ASP400 Clay	NE
21. Morwet DB	NE
22. Alkamide DL-207/S	Severe at 1000 ppm
23. Morwet 3008	Moderate in one rep at 1000 ppm
24. Emery 2270 Methyl Laurate 70	NE
25. Emerest 2301 Methyl Oleate	NE
26. Alkamide DC-212/S	Severe at 1000 ppm
27. Emerest 2209 Methyl Caprylate Caprate	NE
28. Alkamide DIN 295/S	NE
29. Witcamide 511	NE
30. Witconol 14, Polyglycerol-4 Oleate	NE
31. D-Limonene	Moderate in one rep at 1000 ppm
32. Sunspray 8N	NE
33. Safflower oil	NE
34. Corn oil	NE
35. Sunflower oil	NE
36. Peanut oil	NE

[a] NE = no effect.

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Only a few of the inerts were found to be inhibitory and they were inhibitory only at the highest concentration. The oils and some of the other inerts were not miscible in water, therefore their concentrations are approximate.

Effect of wet milling on viability of the fungus

Fungal biomass from flask 3 was homogenized in a blender (Waring®) or with a tissue homogenizer (Tissumizer®, Tekmar Co.) for 10, 40 and 160 seconds. At the end of each run the temperature of the homogenate was measured. The homogenate was diluted and spread-plated to determine colony-forming-units per gram (cfu/g). Colonies were counted after two days incubation of spread plates. When the biomass was milled in the blender there was a significant increase in cfu/g after 40 s and after 160 s there was a trend toward increased cfu/g (Table 2). The temperature of the suspension was approximatley 25°C before milling, and reached 35-36°C after 160 s. When biomass was milled with the Tissumizer® the increase in cfu/g was more rapid and the maximum, reached at 40-160 s, was slightly greater than the maximum achieved with the blender. Heat generation was significantly lower when the Tissuemizer® was used except in one case where one of the two replications at 160 s reached a temperature of 45°C. It is not known why the temperature in that case increased so rapidly, but it could have been due to clogging of the bit or the Tissumizer® probe bearing may have been failing.

Table 2
Effect of milling on viability of M. terrestris shake-flask biomass

Method	Time (s)	Temperature (°C)	Mean Cfu/g
Blender control	0	24-25	$2.8X10^5$
Blender	10	28-29	$3.8X10^5$
Blender	40	30-32	$1.8X10^6$
Blender	160	35-36	$3.2X10^6$
Tissumizer® control	0	24-25	$1.7X10^5$
Tissumizer®	10	25	$1.1X10^6$
Tissumizer®	40	27	$6.0X10^6$
Tissumizer®	160	31-32	$5.5X10^6$
		(45)	$(6.5X10^3)$ [a]

[a] In one of the Tissumizer® treatments the temperature increased to 45°C which may have resulted in the reduced cfu/g observed.

Effect of drying on viability of the fungus

Fungal biomass from Flasks 1 and 2 was homogenized for approximately 10 s and mixed with Barden clay to produce a preparation with a "cookie-dough" consistency. The dough was pressed through a sieve to produce "granules" and the granules were dried in a fume hood (ambient), in a forced air oven at 35°C, or in a fluid bed drier/granulator (Aeromatic, Inc.). Viability was determined by dilution and spread plating methods. After 2-3 days incubation, colonies on plates were counted to determine cfu/g. Percent moisture of each treatment preparation was determined gravimetrically (Table 3).

Table 3
Effect of drying on viability of M. terrestris shake-flask biomass

Method	Time (h)	Moisture (%)	Cfu/g	Corrected cfu/g[a]
Control	0	44	$4.2X10^6$	$7.5X10^6$
Ambient	2	11	$4.3X10^5$	$4.8X10^5$
Ambient	4	2.8	$2.5X10^5$	$2.6X10^5$
Ambient	24	1.8	$2.9X10^4$	$3.0X10^4$
Oven (35°C)	1	4.4	$1.3X10^5$	$1.4X10^5$
Oven (35°C)	2	0.6	$8.5X10^4$	$8.6X10^4$
Oven (35°C)	4	0.9	$1.1X10^4$	$1.1X10^4$
Aeromatic drier	0.17	10	$3.2X10^5$	$3.6X10^5$
Aeromatic drier	0.33	2.6	$8.7X10^4$	$8.9X10^4$
Aeromatic drier	0.5	1.1	$3.8X10^4$	$3.8X10^4$

[a]Cfu/g was corrected for moisture content:

Cfu/g dry wght = (cfu/g wet wght)(100%/(100% - moisture %)).

Drying from 44% moisture to about 10% moisture resulted in a log reduction in cfu. Another log reduction resulted with decrease in moisture to 1-2%. It was also found that the control (no drying) plates were ready for counting after 2 days, but the dried treatments took an extra day to grow on the agar plates.

Mycoleptodiscus terrestris A5 was compatible with most of the tested formulation inerts. Wet milling using a Tissumizer® increased cfu/g to a maximum of approximately $6X10^6$ cfu/g after 40 s. Drying the fungus resulted in significant reduction in numbers of living fungal units.

Formulations development

Four fermentations of the fungus were harvested for use in development of the experimental formulations. The formulations prepared and evaluated were: invert emulsions, extruded granules, suspension concentrates, spray-dried, oil flowable, and fiber. The sources and descriptions of formulation ingredients are listed in Appendix A.

Production of fungal biomass

Biomass of M. terrestris A5 was produced for formulation development in 28 L fermentors. (Batch sheets for each of the four fermentations are in Appendix B). The fermentors were harvested by centrifugation to concentrate the fungal biomass (wet pack). The cfu/g wet pack were determined by standard dilution and spread plating techniques (Table 4). Two of the wet packs were contaminated with significant numbers of bacteria and were used only in preliminary formulation studies.

Table 4
Colony-forming-units of M. terrestris and bacterial contaminants in fermentation wet packs

Wet pack	M. terrestris	Bacterial contamination
21162-35	$9.1X10^6$	$8.3X10^5$
21162-38	$6.8X10^6$	$>2X10^3$
21162-59	$9.5X10^6$	$3.5X10^6$
21162-60	$3.6X10^6$	0 detected

Invert emulsions

An invert emulsion is a stable suspension of an aqueous phase in an oil phase. Mayonnaise is an example of an invert emulsion. A series of inverting agents were screened for their ability to form a corn oil/water

invert emulsion. The inverting agents were Witconol 14, Alkamide DIN, Alkamide DC 212/S, Witcamide 511, Alkamide DS 280/S, Alkamide 207/S, and Emid 6545.

Approximately 100 g of either 5 or 10% inverting agent in corn oil was prepared. The oil phase was mechanically stirred while small increments of tap water were added. Most preparations did not invert, however, if an invert emulsion formed, the volume of water added was noted. Most of the successful inverts occurred after approximately 40 g of water was added to the oil phase.

Six oils were screened for their effects on the fungus. Slurries of Mycoleptodiscus in each of the oils were prepared. Each slurry contained approximately 10 g wet pack in 40 g oil. In all slurries the fungus settled out of suspension. The slurries were plated by standard methods to determine cfu/g (Table 5).

Table 5
Compatibility of wet pack in various oil slurries

Batch	Oil	Cfu/g
60374-04-S1	Corn Oil	Approx. 10^4
60374-04-S2	Emery 2231 (methyl canolate)	6.0×10^3
60374-04-S3	Emery 2301 (methyl oleate)	3.8×10^4
60374-04-S4	Sunflower Oil	5.9×10^4
60374-04-S5	Sun Spray 8N	2.6×10^5
60374-04-S6	d-Limonene	0

The Sun Spray 8N slurry had the greatest cfu/g. d-Limonene caused a loss in viability to below the detection level of the plating technique (detection limit = $2.5X10^2$). The corn oil slurry did not disperse in the diluent. A drop of a surfactant (Tween 80®) was added to the first tube in the dilution series to aid dispersion. This appeared to increase dispersion of the corn oil slurry, but the cfu/g from the dilutions and spread plating were not as reliable for this slurry as for the others.

To increase the stability of suspensions, two aqueous slurries containing 1% clay were prepared. Each contained approximately 60 g aqueous phase and 12 g of wet pack. Both clays thickened the suspension and reduced the rate at which the fungus settled out of suspension. Cfu/g were determined (Table 6).

Table 6
Effect of clays in aqueous suspension on viability of M. terrestris wet pack

Batch Number	Clay	Cfu/g
60374-05-S14	Volclay Bentonite HPM-20	5.4×10^4
60374-05-S15	Attagel 50	4.3×10^5

The Attagel 50 suspension had greater cfu/g.

Inverting agents were screened by blending approximately 5 g inverting agent with 40 g corn oil and 10 g wet pack. Each batch was sheared briefly with the Tissumizer®. Cfu/g were determined (Table 7).

Table 7
Viability of M. terrestris wet pack in corn oil amended with various inverting agents

Batch Number	Inverting Agent	Cfu/g
60374-05-S7	Witconol 14	3.8×10^5
60374-05-S8	Witcamide 511	1.6×10^4
60374-05-S9	Emid 6545	3.7×10^4
60374-05-S10	Alkamide DC-212/S	None Detected
60374-05-S11	Alkamide DIN 295/S	None Detected
60374-05-S12	Alkamide DL 207/S	None Detected
60374-05-S13	Alkamide DS 280/S	None Detected

Witconol 14 in corn oil yielded the greatest cfu/g. Corn oil was used even though it did not give the greatest cfu/g in a previous experiment (Table 5). It was believed that any oil would be suitable for initial evaluation of inverting agents. In a later experiment various oil/inverting agent combinations were compared for selection of the best oil/inverting agent system.

Based on the above data, the final series of screening batches was prepared. Each batch was formulated with 68 g oil, 20 g wet pack and 12 g inverting agent. The mixtures were sheared briefly with the Tissumizer® and cfu/g were determined (Table 8).

Table 8
Viability of M. terrestris in variuos oil/inverting agent combinations

Batch Number	Inverting Agent	Oil	Cfu/g
60374-12-S23	Witconol 14	corn oil	7.5×10^4
60374-12-S24	Witcamide 511	corn oil	5.0×10^3
60374-12-S25	Emid 6545	corn oil	5.0×10^3
60374-12-S26	Witconol 14	Emery 2301	1.6×10^5
60374-12-S27	Witcamide 511	Emery 2301	4.0×10^4
60374-12-S28	Emid 6545	Emery 2301	5.0×10^3
60374-13-S29	Witconol 14	sunflower oil	8.5×10^4
60374-13-S30	Witcamide 511	sunflower oil	2.0×10^4
60374-13-S31	Emid 6545	sunflower oil	2.0×10^4
60374-13-S32	Witconol 14	canola oil	7.0×10^4
60374-13-S33	Witcamide 511	canola oil	2.5×10^4
60374-13-S34	Emid 6545	canola oil	2.0×10^4
60374-13-S35	Witconol 14	Sunspray 8N	1.6×10^5
60374-13-S36	Witcamide 511	Sunspray 8N	2.5×10^4
60374-13-S37	Emid 6545	Sunspray 8N	3.5×10^4

Combinations of Witconol 14 and either Emery 2301 (methyl oleate) or Sunspray 8N yielded the greatest number of cfu/g.

Five formulations were prepared to evaluate the effect of altering the concentration of the inverting agent. Witconol 14 at 6, 8, 10, 12, and 14 g/100 g formulation was mixed with Emery 2301 (methyl oleate) and wet pack (Table 9). The preparations were blended, then sheared briefly with the Tissumizer®. While the oil phase was being mechanically stirred with a propeller stirrer, tap water was slowly added. When maximum viscosity was attained, the quantity of water added was noted and a visual assessment of the invert's viscosity was made. The invert was added to water to assess dispersibility. A low degree of dispersibility was considered desireable, indicating relative stickiness.

Table 9
Optimization of Witconol 14 (inverting agent) in invert emulsion formulations of M. terrestris

Ingredient	Quantity (g)				
Wet pack	20	20	20	20	20
Emery 2301	74	72	70	68	66
Witconol 14	6	8	10	12	14
Total	100	100	100	100	100
mL water at maximum viscosity	100	100	75	50	50
Relative viscosity	low	low+	moderate	high	high
Disperses in water	no	no	slight	yes	yes

As the quantity of inverting agent increased, the water required to form an invert emulsion decreased and viscosity of the resulting emulsion increased. However, invert emulsions with 10-14 g inverting agent dispersed in water, indicating reduced stickiness. Thus, a low concentration of inverting agent is preferred.

All of the invert emulsions floated when added to water. It is believed that air became entrapped during the inverting process, resulting in the observed buoyancy. Efforts were directed at increasing the invert's density to cause it to sink. The effect of increasing the concentration of wet pack in the oil phase was evaluated (Table 10).

Table 10
The effect of increasing the amount of wet pack in invert emulsions on formulation buoyancy

Ingredient	Batch-->	Quantity (g) 60444-02-B38	60444-02-B39[a]
Wet pack		30	40
Emery 2301		64	54
Witconol 14		6	6
Total		100	100
mL water to invert		50-75	90
buoyancy in water		floated	floated

[a]Formulation was very viscous before inverting.

Adding more wet pack did not decrease buoyancy. The effect of adding clays to increase density was studied (Table 11).

Table 11
Effect of clay on buoyancy of invert emulsion formulations

Ingredient	Batch-->	Quantity (g) 60444-02-B40	60444-02-B41	60444-02-B42[a]	60444-02-B43[b]
Wet pack		20	20	20	20
Emery 2301		69	64	64	65
Witconol 14		6	6	6	6
Attagel 50		5	10	- -	- -
Barden Clay AG-1		- -	- -	10	- -
Bentone 38		- -	- -	- -	5
Total		100	100	100	100
mL water to invert		75	80	75	50
buoyancy in water		floats	floats	partial[c]	partial[c]

[a]Invert had low viscosity and partially dispersed in water.
[b]Emulsion was very stiff.
[c]Invert separated into one component which sank and one which floated.

Attagel 50 did not have a noticeable effect on buoyancy. Barden clay AG-1 and Bentone 38 preparations separated in water with one component sinking and the other floating.

The final invert was prepared in a two part system. The oil phase was prepared as described previously. Glycerin was added to the water phase. The two phases were combined resulting in an invert emulsion with low viscosity and reduced buoyancy (Table 12).

Table 12
Two phase invert preparation of batch 60444-03-B44

Ingredient	Quantity (g)
Oil Phase	
Wet pack	20
Emery 2301	67
Witconol 14	6
Bentone 38	1
Subtotal	94
Water Phase	
Water	60
Glycerin	30
Subtotal	90

It was not possible to control air entrainment on a lab scale. The addition of various clays to the formulation reduced buoyancy despite the high degree of air entrainment. It may be possible to control air entrainment at the time of application in the field by pumping the aqueous and oil phases simultaneously through the application hose without prior mixing. The invert emulsion is actually formed in the pump. If the pump is sealed and flow rate is adequate there should be little or no air entrainment.

Extruded Granule Formulations

Extruded granules are produced by passing a formulation "dough" through a small orifice. The extruded strings of dough can be cut to give granules of various sizes. The Formulations Group envisioned a granule that would float initially and as it hydrated would slowly descend through the water and come into contact with the submerged weed. In the initial phase the feasibility of the extruded granule system for M. terrestris wet pack was demonstrated. In Phase 2 the focus was to produce granules that floated, that broke apart and sank, that were sticky, and that would ascend through an effervescing action.

Extruded granules - Phase 1

Extruded granules were prepared using the LCI Benchtop Extruder. The ingredients were combined and blended until a uniform powder was obtained (Table 13). The powder was sprayed with water to produce a dough that could be extruded. Batch 60374-07-S18 was extruded through a 2.0 mm screen. Batches 60374-10-S21 and 60374-10-S22 were extruded through a 1.0 mm screen. Samples were taken before and after drying overnight at room temperature and cfu/g were determined (Table 14).

Table 13
Extruded granule ingredients - Phase 1

	Quantity (g)		
Ingredient Batch-->	60374-07-S18	60374-10-S21	60374-10-S22
Wet pack	40	51	54
Corn starch	10	9	10
Kelsan	1	1	1
Barden clay AG-1	21	--	--
Volclay Bentonite HPM-20	56	--	--
Attagel 50	--	41	--
Lattice NTC	--	--	41

Table 14

M. terrestris cfu/g in Phase 1 extruded granule formulations

Batch	Description	Cfu/g
60374-07-S18-1	wet granules	2.3×10^4
60374-07-S18-2	air-dried granules	2.0×10^5
60374-10-S21-1	wet granules	0 detected[a]
60374-10-S21-2	air-dried granules	1.8×10^4[a]
60374-10-S22-1	wet granules	0 detected[a]
60374-10-S22-2	air-dried granules	0 detected[a]

[a]Bacterial contamination interfered with growth and colony counting.

The dried granules contained greater cfu/g than the wet granules in this experiment. This was not expected because of the deleterious effect of drying. As was shown in a preliminary study (Table 3) and will be seen in data to follow in this report, the general result of drying was decreased viability of the fungus. Bacterial contamination of formulations 60374-10-S21 and -S22 interfered with colony counting and may have inhibited fungal growth. The dried granules sank immediately when added to water.

Extruded granules - Phase 2

The ingredients for each formulation were weighed into a 4 L beaker and mixed by hand with a rubber spatula to a uniform mixture. When it was necessary to add water to the formulation, it was sprayed on with a hand held spray bottle and then mixed with the spatula until the appropriate moisture level was obtained. One formulation was mixed with a blender (Waring) to determine the effect on viability of the fungus.

The material was placed into the LCI benchtop extruder equipped with a 1.0 or a 1.2 mm screen. Slight pressure was applied from the top to keep the material in contact with the blades. As the granules were extruded through the screen, they were cut with a spatula to a uniform length. The granules were placed in a fume hood with the blower on to air-dry overnight. The granules were then screened through 10 and 20 mesh screens. Twenty-two extruded granule formulations were made (Table 15).

Table 15
Extruded granule formulations, batches 60450-1 through 60450-15, 60450-17 through 60450-22 and 60450-25

Ingredients Batch-->	Quantity (g) 60450-1	60450-2	60450-3	60450-4	60450-5
Wet pack	40	40	40	28.8	32
Lattice NTC	40	--	--	--	--
Attagel 50	--	40	--	--	--
Sea Spen PF	--	--	25	--	--
Hi-Sil 233	--	--	15	--	--
1895 Dextrin	--	--	--	28.8	--
Celite 266	--	--	--	22.4	32
Gelex® Instant Starch	--	--	--	--	16
Water	5	17	42	--	--

(Table 15. continued)

Ingredients Batch-->	Quantity (g) 60450-6	60450-7	60450-8	60450-9	60450-10
Wet pack	40	40	24	24	24
Avicel pH-102	40	--	--	--	--
Cellulose gum 7M	--	40	--	--	--
Lattice NTC	--	--	32	32	--
Sodium bicarbonate	--	--	16	--	--
Citric acid	--	--	8	--	--
Celite 266	--	--	--	24	32
Sea Spen PF	--	--	--	--	46
Aerosil	--	--	--	--	10
Water	17	--	6.5	41	74

(continued)

(Table 15. continued)

Ingredients	Batch-->	Quantity (g) 60450-11	60450-12	60450-13	60450-14	60450-15
Wet pack		24	24	24	24	24
Lattice NTC		24	24	--	--	--
Celite 266		9.6	8	--	--	--
Nadex 772		32	--	--	--	--
Nadex 360		--	24	25.6	16	20
Sea Spen PF		--	--	25.6	--	--
Aerosil R972		--	--	4.8	--	0.8
Sodium bicarbonate		--	--	--	16	--
Citric acid		--	--	--	8	--
Jaguar 2228		--	--	--	--	35.2
Water		--	8	49	6	>80

(Table 15. continued)

Ingredients	Batch-->	Quantity (g) 60450-17	60450-18	60450-19	60450-20	60450-21
Wet pack		24	32	24	24	32
Cellulose gum 7M		16	4	--	--	--
Nadex 360		39.2	--	25.6	25.6	8
Aerosil R972		0.8	1.6	4.8	4.8	4.8
Attagel 50		--	42.4	--	--	--
Sea Spen PF		--	--	25.6	25.6	8
Celite 266		--	--	--	--	9.6
Water		42	20	70	70	55

(continued)

(Table 15. concluded)

Ingredients	Batch-->	Quantity (g) 60450-22	60450-25
Wet pack		32	32
Nadex 360		9.6	7.8
Aerosil R972		4.8	5.4
Sea Spen PF		16	18
Celite 266		9.6	7.8
Cellulox gum 7M		8	--
Cellulose gum 7M		--	9
Glycerin		--	10
Water		45	15

Clays, starches and silicas were used in the first batches to help the formulation float and break apart in the water. Granules were added to water and behavior was observed (Table 16).

Table 16
Behavior in water of extruded granule formulations

Formulation	Observed behavior in water
60450-1	Half of granules sank immediately; the rest floated for 2-3 min, then broke apart and sank.
60450-2	Almost all of the granules sank immediately and quickly broke apart into fine particles.
60450-3	Half of the granules sank immediately, but descended slowly. The granules broke apart fairly quickly. The floating granules sank after about 5 min.
60450-4	Half of granules sank immediately, the rest within 45 sec. The granules did not break apart.
60450-5	A little more than half of the granules sank immediately. Some granules were floating after 5 min. Granules did not break apart.
60450-6	About 75% sank immediately. Granules were very dense, did not break apart.
60450-7	Granules sank immediately. They were very gummy and sticky, and when dispersed were colloidal.
60450-8	Half sank immediately. They broke apart very slowly.
60450-9	Half sank immediately. They broke apart very slowly.
60450-10	All granules floated and they wetted out very, very slowly.

(continued)

(Table 16. concluded)

Formulation	Observed behavior in water
60450-11	All sank and broke apart very slowly.
60450-12	Half sank immediately. Floating granules gradually broke apart.
60450-13	All floated. Granules broke apart slowly, descended slowly, and in some cases fragments rose back to the surface.
60450-14	Half sank immediately. The granules wetted out faster than 60450-13. Floating granules sank slowly.
60450-15	All floated. Large sticky looking globules formed.
60450-17	Half sank immediately. The granules quickly became gummy and sticky.
60450-18	Half sank immediately. Granules broke apart.
60450-19	All floated. After 3-4 minutes granules began to descend slowly.
60450-20	All floated. After 3-4 minutes granules began to descend slowly.
60450-21	All floated. After 2-3 minutes granules began to descend very slowly.
60450-22	All floated. After 5 minutes granules began to descend slowly.
60450-25	All floated. After 5 minutes granules began to descend.

The activity of granules in water covered a wide range. Granules of some formulations sank immediately. Some floated for a few seconds and then sank. Some granules broke up quickly while some never broke apart. Celite 266 and Aerosil R972 improved granule buoyancy. The combination of Nadex 360, an insoluble starch, and SeaSpen PF provided binding properties that allowed the granule to slowly hydrate, eventually break apart and slowly sink to the bottom.

Sodium bicarbonate and citric acid were added to two formulations to produce an effervescing granule. The granules did not show signs of fizz or bubbles when added to water. No attempts to further improve this formulation were made.

After achieving a granule that floated and broke apart slowly, additives to increase stickiness of the formulation were tested. Of the binders and thickeners tested, Cellulose Gum 7M produced the best result.

Throughout the formulation process, those granules with the best physical properties were plated to determine relative viability of the fungus. Whole granules were sprinkled on a nutrient agar, incubated at 30°C and growth from the granules was evaluated after 24-72 h. It became apparent that at some point during formulation viability of the fungus declined. Based on previous experience with the negative effects of drying, glycerin was added to the most promising formulation to prevent excessive moisture loss from the granules. Viability of the fungus in glycerin-amended granules was much improved. The best extruded granule (batch 60450-25) contained wet pack (35%),cellulose gum 7M (10%), SeaSpen PF (20%), Celite 266 (8.7%), Aerosil R972 (6.0%), Nadex 360 (8.7%), and glycerin (11.1%).

Spray-dried Formulations

Spray-dried powders are an economical formulation used extensively in the pesticide industry. A spray-drier is used to flash off the moisture from a slurry of the product as it is sprayed into the spray tower. Four batches of spray-dried powder were prepared (Table 17).

Table 17
Ingredients used in spray-dried formulations

	Quantity (g)			
Ingredient Batch-->	60374-06-S16	60374-06-S17	60374-08-S19	60374-09-S20
Wet pack	28.5	26.0	54.5	32.0
Volclay Bentonite HPM-20	21.7	18.4	20	10
Corn Starch	3.5	6.5	10	5
Barden Clay, AG-1	- -	9.7	9	- -
Kelsan	- -	- -	1	1
Polyfon H	- -	6.1	- -	- -
Surfynol 104S	- -	2.1	- -	- -
Pluronic P127	- -	- -	- -	2

Batch 60374-06-S16 was blended with approximately 200 g of tap water and stirred using a stir plate. Some of the wet pack in the slurry did not disperse. It was spray-dried using a Buchi 190 Mini Spray-Drier, Model 8 190. Initially, the tower temperature was set at 60°C (Batches 60374-06-S16-1 and -S16-2). Later, the temperature was raised to 80°C (Batches 60274-06-S16-3 and -S16-4). Throughout the runs at both temperatures a dark residue, appearing very similar to the wet pack, collected in the spray tower. The powder collected in the product receiver appeared to be much lighter in color. Batch 60374-06-S17 was prepared much as was 60374-06-S16, but was sheared with the Tissumizer® to increase the dispersion of the wet pack. It was spray-dried at 80°C with results very similar to the previous run.

Batch 60374-08-S19 was milled in two installments using a type B, size 01 intermittent attritor. The first installment was milled for 15 minutes and the second for 18 minutes. This batch was spray-dried at 80°C. Results were similar to the previous runs. Batch 60374-09-S20 was first sheared with the Tissumizer®, then milled using an attritor for 20 minutes. Milling significantly reduced the amount of undispersed wet pack. It was spray-dried as previously. Samples of all batches, taken at various points, were evaluated for cfu/g (Table 18).

Table 18
Viabliity of M. terrestris in samples of spray-dried formulations

Sample	Temperature (°C)	Sample Description	Cfu/gm
60374-06-S16-1	60	wet paste from tower	3.9×10^5
60374-06-S16-2	60	powder	0 detected
60374-06-S16-3	80	wet paste from tower	1.9×10^5
60374-06-S16-4	80	powder	0 detected
60374-06-S17-1	80	powder	0 detected
60374-06-S17-2	80	wet paste from tower	0 detected
60374-08-S19-1	--	slurry from attritor	2.5×10^3
60374-08-S19-2	80	rinsed from tower	0 detected
60374-08-S19-3	80	powder	0 detected
60374-09-S20-1	--	before milling	1.5×10^4
60374-09-S20-2	--	milled 20 minutes in attritor	0 detected
60374-09-S20-3	80	powder	0 detected

It will be difficult to prepare a satisfactory spray-dried product without increasing dispersibility of the wet pack. Samples of batch 60374-06-S16 taken from the spray tower had acceptable counts. No cfu were detected in the powder collected at the receiver. No nozzle clogging problems were noted. Apparently, the wet pack was not sufficiently dispersed in the spray slurry to allow it to dry and be carried into the product receiver with the other formulation ingredients. Instead, it was deposited on the sides of the spray tower. The final two spray-dried formulations were milled using the attritor to resolve that problem. However, the extra shear reduced cfu of the fungus to below the detection limit. It may be possible to process the wet pack to be more dispersible. When observed microscopically (400X) little or no fungal structures were observed in any of the dry powders.

The temperatures required to dry fungal slurries were relatively high from a biological standpoint. The drying temperatures used in the spray-drying effort were the lowest that would effectively evaporate the water from the wet pack slurries. Evaporative cooling may reduce the amount of direct heat exposure to the fungus.

Suspension Concentrate Formulations

During studies to develop an extruded granule formulation additives to make the granules sticky so that they would adhere to the weed were evaluated. A spin-off of this research was suspension concentrate formulations based on honey and corn syrup. Wet pack and dry ingredients were mixed in a blender. The syrup or honey was placed in a glass beaker and mixed with a dispersator as the wet pack/dry mixture was added slowly. The suspension was mixed for 10 min to ensure homogeneity.

When added to water the suspension concentrates became stringy and clung onto leaves of the weed as they descended. The honey formulation sank rapidly while the syrup sank at a slower rate. After approximately two weeks the wet pack particles had begun to settle out of the honey formulation while the wet pack remained suspended in the syrup formulation. The syrup formulation consisted of wet pack (4.5%), Celite 266 (3.1%), Aerosil R972 (1.4%), and Light Karo Syrup (91%). In both formulations viability decreased when stored at approximately 5°C. Increasing the level of wet pack in the formulation from the current 4.5% should increase cfu/g of the syrup formulation.

Oil Flowable Formulation

A single oil suspension concentrate type formulation, batch 60444-03-B44 was made. Emery 2301 (61.2 g), Bentone 38 (0.8 g), Trycol 6962 (9.0 g), and wet pack (31.5 g) were combined and sheared using the Tissumizer®. Drops of the formulation were plated to determine viability of the fungus. The fungus did not grow from the oil suspension.

Fiber Formulation

Preliminary experiments were done with a formulation based on fibers impregnated with the fungus. A fibrous preparation of the fungus could become entangled in the weed allowing prolonged contact to increase the chances of infection. Cheesecloth was shredded, autoclaved and added to sterile, plastic, tissue culture flasks. An agar culture of the fungus was blended in liquid nutrient medium and the resulting slurry was added to the flasks until the cheesecloth was saturated. The flasks were incubated at room temperature under a 12 h photoperiod. After 9 wk the cheesecloth, covered with dark growth of the fungus, was removed from the flask and dried overnight at ambient conditions. Observed microscopically there appeared to be degradation of the cotton fibers of the cheesecloth. When plated on water agar growth of the fungus was very slow. One month after plating, conidia of M. terrestris were produced in sporodochia that were scattered over the agar surface. This type of formulation could be developed further if a suitable fibrous substrate which can be used in submerged fermentation is available.

Evaluation of selected formulations in water with Hydrilla

Formulations of the fungus were selected for further evaluation based on viability and behavior in water. Ten formulations were observed in a Hydrilla test tube assay. The four best were then tested in a moving water assay with the weed.

Test tube assay

A sprig of Hydrilla was placed in 10 cm deep water in a 2.5 cm diameter plastic centrifuge tube. The test formulation was added (a few granules or drops depending on the formulation type), and observations of behavior were made (Table 19.)

Table 19
Behavior of selected formulations of M. terrestris in a Hydrilla test tube assay

Formulation	Characteristics
60450-19	Granules floated. As they hydrated the granules became fibrous/gelatinous/spongy and descended through the water column to catch on the leaves of the weed. Formulation did not cling to leaves if shaken.
60450-20	Behaved as 60450-19.
60450-21	Behaved as 60450-19.
60450-22	Behaved similarly to 60450-19, however, some particles of the granules remained suspended. Some of the sunken fragments ascended to the surface.
60450-25	Behaved as 60450-22.
60450-23	Globules sank rapidly to bottom leaving a string-like trail that dissolved in the water.
60450-24	Very viscous drops fell through sprig to bottom, catching in axils as they descended. Drops in water dispersed after several minutes. Water was cloudy white with a surface film. After standing several hours, a white coating was noted on the undersides of leaves.
60444-02-B43	Very stable invert, floated on the surface. After 1 month the invert globule remained intact at surface covered with fungal growth.
60444-03-B44	A loose invert, it floated and then dispersed rapidly. Particle of fungus released from the emulsion to sink to the bottom, while an oily film remained at the surface.
60444-03-B45	Behaved as 60444-03-B44.

Flowing water assay

The moving water assay system consisted of a 1.2 m length of rain gutter with a water inlet at one end and an outlet at the other end. The rate of flow was not measured, but there was a slight postive flow from the inlet end to the outlet end. Hydrilla sprigs approximately 8-10 cm in length were stuck into sand about 20-30 cm from the outlet end. Additional sprigs were laid across the surface of the water and held in place by the sprigs stuck in the sand. The treatments were added approximately 10 cm upstream or directly into the Hydrilla and behavior was observed (Table 20).

Table 20
Behavior of selected formulations of M. terrestris in a flowing water Hydrilla assay

Formulation	Characteristics
60450-22	Some of the granules sank and some floated. The floating granules passed over the weed or were caught by leaves sticking above the surface. Sunken granules hydrated and floated submerged or at the surface toward the weed. Submerged granules tended to float through the sprigs of Hydrilla while surface granules clung to the emerged leaves. The clinging granules could be easily dislodged by agitation. Over several minutes the granules fragmented into small particles.
60450-24	The syrup formulation was very viscous. The drops fell to the bottom and slowly dissolved. When dropped directly over the Hydrilla it draped over leaves as it descended. There was a white coating of leaves at the surface.
60444-02-B43	This stable invert floated unless forceably injected into the water around the weed where the strings of formulation became entangled in the sprigs. There was no sign of invert breakdown.
60444-03-B44	An oily film developed at the surface. Globules of fungus in oil were entrapped in the sprigs of Hydrilla. Oily globules adhering to emergent leaves could be easily dislodged by agitation.

Conclusions and Recommendations

The most promising of the formulations were the extruded granules and the invert emulsions. While progress was made, all of the formulations require improvement. The invert emulsions had good initial vigor and viability, but they float at the water's surface. Preapplication mixing of water and oil phases without the introduction of air may produce a product of neutral buoyancy. Alternatively, a floating invert could be applied beneath the surface of dense mats of vegetation, float up and become entrapped by the target weed. The introduction of oil into the aquatic environment may be a concern, however, the oils used are biodegradable.

As with the invert emulsions, progress was made with formulations of extruded granules. The best granules became spongy and, because some of the material floated and some sank, the possibility of contacting the target was enhanced. The problem with low viability after drying was addressed by the addition of glycerin. Perhaps further improvements in viability and stability could be achieved by optimizing the level of glycerin or by substituting oils or other humectants for glycerin. The amount of wet pack in the granule formulations could be increased to enhance infection potential. Although the addition of Cellulose Gum 7M did increase stickiness, this characteristic needs improvement. The syrup suspension concentrate appeared to be sticky and this type of formulation could be studied further. In vivo studies to be done at WES will increase the data base for making decisions on which formulation types should be pursued.

In the current studies there was no long term viability testing, however, viability platings of some of the granules, suspension concentrates, and invert emulsions were repeated. Viability and vigor declined over a matter of weeks even when the experimental formulations were refrigerated (approximately 5°C).

The most likely reason for decline in viability was that the fungus wet pack was mycelial. A formulation based on structures of the fungus more suited to survival (e.g., conidia, chlamydospores, sclerotia) should increase stability of the product. The best formulation will not result in an effective product if the active ingredient, the fungus, is not optimized. Future development efforts should include strain selection, enhancement of survival/stability and infectivity in production, and optimizing fermentation. The WES collection of M. terrestris isolates should be screened for characteristics such as spore production in submerged culture, ability to tolerate dessication, temperature and uv tolerance, virulence, rapid growth in culture, and culture yield. After the best isolates have been selected experiments to determine methods of production which would yield fungal propagules with greater stability than filamentous hyphal cells should be completed. Factors such as temperature, light, nutrients, and agitation/aeration should be studied to determine their effects on production of conidia, chlamydospores and sclerotia. Media additives or other stimuli might also be used to enhance propagule survivability. Optimizing fermentation will involve fine tuning medium components, inoculum age and volume, and agitation. Production in the 250 or 500L fermentors is necessary to predict scale up success in production scale fermentors. Downstream processing methods for the fermentor biomass will require development. After the most effective isolate has been selected and biomass production methods have been optimized, further improvements in the formulations should be made.

Appendix A
Sources and Descriptions of Formulation Ingredients

Ingredient	Source	Description
1. SeaSpen PF	FMC Corp.	viscosity modifier
2. Nadex 360	National Starch and Chemical Co.	starch/viscosity modifier
3. Cellulose Gum 7M	Aqualon	viscosity modifier
4. Aerosil R972	Degussa Corp.	viscosity modifier/buoyancy enhancer
5. Glycerin	Fisher Scientific	humectant
6. Celite 266	Celite Corp.	
7. Citric Acid	Fisher Scientific	effervescing agent
8. Sodium Bicarbonate	Fisher Scientific	effervescing agent
9. Nadex 772	National Starch and Chemical Co.	starch/viscosity modifier
10. Lattice NTC	FMC Corp.	viscosity modifier
11. Avicel PH-102	FMC Corp.	viscosity modifier
12. Attagel 50	Engelhard Corp.	swelling clay
13. 1895 Dextrin	American Maize-Products Co.(Amaizo)	starch/viscosity modifier
14. Gelex®Instant Starch	American Maize-Products Co.(Amaizo)	starch/viscosity modifier
15. Hi-Sil 233	PPG	viscosity modifier/buoyancy enhancer
16. Jaguar 2228	Rhone-Poulenc Inc.	thickener/guar gum
17. Clover Honey	W. Stoller's Honey, Inc.	carrier
18. Light Karo Syrup	Best Foods, CPC International Inc.	corn syrup/carrier
19. Corn starch	Cream Brand, Dial Corp.	viscosity modifier
20. Witconol 14	Witco, Inc.	inverting agent
21. Witcamide 511	Witco, Inc.	inverting agent
22. Alkamide DIN	Rhone-Poulenc Inc.	inverting agent
23. Alkamide DC 212/S	Rhone-Poulenc Inc.	inverting agent
24. Alkamide DS 280/S	Rhone-Poulenc Inc.	inverting agent
25. Alkamide 207/S	Rhone-Poulenc Inc.	inverting agent
26. Emid 6545	Henkel Corp.	inverting agent

(continued)

(Appendix A, concluded)

Ingredient	Source	Description
27. Canola oil	Crisco brand, P&G	oil
28. Corn oil	ADM	oil
29. Emery 2231	Henkel Corp.	methyl oleate
30. Emery 2301	Henkel Corp.	methyl oleate
31. Sunflower oil	Wesson	oil
32. Sun Spray 8N	Sun Refining and Marketing Co.	oil
33. d-Limonene	Florida Chemical Co.	citrus oil
34. Volclay Bentonite HPM-20	American Colloid Co.	clay
35. Barden clay AG-1	Huber, Inc.	clay
36. Kelsan	Kelco, Inc.	viscosity modifier
37. Polyfon H	Westvaco, Inc.	lignosulfonate dispersant/carrier
38. Surfynol 104S	Air Products and Chemicals, Inc.	wetting agent
39. Pluronic P127	BASF, Inc.	wetting agent
40. Bentone 38	Rheox Corp.	organophilic clay
41. Trycol 6962	Henkel Corp.	surfactant
42. Curity® cheesecloth	The Kendall Co.	cotton fiber
43. Mycoleptodiscus	Ricerca, Inc.	fungal biomass

A2

Appendix B
Fermentation Batch Sheets

B1

FERMENTATION RECORD AGICHEMMYCOID Page 1 of 2

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

FERMENTATION Mycoleptodiscus FERMENTOR: 28L-1 DATE: 10-6-94
LOT NUMBER (CUMULATIVE): R94014 PROJECT NUMBER: 6191 RTO 5776 BATCH #: Mt-1
CULTURE#: Mt AS FERMENTATION STAGE: 1° SEED. 2°SEED. 3° SEED. or (PRODUCTION) (circle one)
VESSEL DESCRIPTION VESSEL SIZE: 28 L TANK COMPOSITION: SS # OF BAFFLES: 4 # OF IMPELLERS: 2
OF BLADES: 6 BLADE TYPE RUSHTON IMPELLER DIST.. BOTTOM: 13.5" . MIDDLE: 8.5" . TOP: NONE.
SPARGER. TYPE: RING DIAMETER: 3.5" POSITION: CENTER, UNDER BOTTOM IMPELLER INITIALS: RTO
MEDIUM DESIGNATION: RICHARD'S MODIFIED V-8 326TH

INGREDIENT	GMS/LITER	GMS/ 20 L	MATERIAL LOT #	PERFORMED BY:	REVIEWED BY:
DEXTROSE	10	200	Spindler D31992	RTO	RTO
KNO_3	10	200	Fisher 901060A	RTO	RTO
$CaCO_3$	3	60	Fisher 923342	RTO	RTO
V-8 JUICE	0.2L/L	4 LITERS	Campbells CU232B 3M6	RTO	RTO
SAG 471	0.1 ml/L	2 ml	Union Carbide 0002RN07291	RTO	RTO
		RTO			
		10-6-94			

BATCHING INSTRUCTIONS

1. ADD 10 LITERS D.I. WATER TO THE FERMENTOR.
2. SLOWLY ADD THE INGREDIENTS IN THE ORDER LISTED ABOVE. MAKING SURE THEY MIX COMPLETELY.
3. ADJUST PH TO — WITH —. PRE-ADJ. PH: NA POST-ADJ PH: — WITH — MLS
4. ADJUST THE VOLUME IN THE FERMENTOR TO 19.5 LITERS WITH D.I. WATER.
5. STERILIZE THE FERMENTOR FOR 30 MINUTES AT 121 °C. TOTAL HEATING TIME = 2hr
6. ADD — LITERS OF — TO THE FERMENTOR ASEPTICALLY FOLLOWING STERILIZATION.
7. ADD STERILIZED ANTIFOAM (SAG 471) AS REQUIRED TO CONTROL FOAMING.

FINAL VOLUME 20 LITERS = BATCH VOLUME 19.5 L + CONDENSATE VOLUME 0 L + INOCULUM VOLUME 0.5 L

INOCULATION

INOCULUM IDENTIFICATION NUMBER: M.T. NAME: Mycoleptodiscus terrestris (AS) AGE: 72H
APPEARANCE: Orange/Red. liquid GROWTH: Fair (PCV, CFU, CPE, OD, ETC.)
INOCULUM PH: 7.4 EXPECTED PH: 7-9 PURITY: OK BY TSA+MICRO INITIALS: RTO

OPERATING CONDITIONS — CHANGES DURING RUN:

TEMPERATURE: 25 °C. NO CHANGES
AERATION: 20 LPM. NO CHANGES
AGITATION: 300 RPM. NO CHANGES
BACK PRESSURE: 10 PSI.
PH — - — WITH —.
ADDITIONS. SHOTS. FEEDS. TO BE ADDED.
MAINTAIN — % DISSOLVED OXYGEN.

COMMENTS/OBSERVATIONS

PERFORMED BY: Richard T. Donnelly 10-6-94 DATE REVIEWED BY: [signature] 10/12/94 DATE

FERMENTATION DATA

Page 2 of 2

FERMENTOR: 28L-1 LOT NUMBER: R94014 DATE: 10-6-94

BATCH NUMBER: Mt-1 PROJECT NUMBER: 6191 CULTURE: Mt (A5)

POST-STER. pH: 6.0 POST-INOC VOLUME: 20L INITIALS: RTO

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

DATE 1994	TIME	AGE (HRS)	TEMP. (°C)	AIR (LPM)	PRESS. (PSI)	AGIT. (RPM)	INST. pH	MEASURED pH	%DISS. OXYGEN	ASSAY RESULT	COMMENTS: PCV, ADDITIONS, ETC.	OPERATOR
10-6	1500	0	28	20	10	300	—	6.0	—	—	—	RTO
10-7	0700	16	28/25	20	10	300	—	6.3	—	—	—	RTO
10-10	0700	88	25	15/20	10/15	300	—	8.2	—	—	—	RTO
10-11	0700	112	25	19/20	15/10	300	—	8.6	—	—	Changing made of form.	RTO
10-11	1300	118	25	20	10	300	—	8.6	—	—	Harvested	RTO

RTO
10-11-94

HARVEST PROCEDURES

HARVEST AGE: 118 HRS HARVEST pH: 8.6 HARVEST VOLUME: 20L HARVEST ASSAY: NA

HARVEST PURITY: OK BY TSA + MICRO INITIALS: RTO

REQUESTED TREATMENT AT HARVEST: Cent. for solids

DELIVER WHOLE BEER, BROTH OR (SOLIDS) (CIRCLE ONE) TO: Bill Hansen YIELD: 1.4 Kg

COMMENTS:

PERFORMED BY: Robert T. O'Donnell DATE: 10-11-94

REVIEWED BY: [signature] DATE: 10/12/94

FERMENTATION RECORD AGICHEM/MICRO Page 1 of 2

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

FERMENTATION Mycoleptodiscus FERMENTOR: 28L-2 DATE: 10-6-94
LOT NUMBER (CUMULATIVE): R94015 PROJECT NUMBER: 6191 FR H 5746 BATCH #: Mt-2
CULTURES: Mt (AS) FERMENTATION STAGE: 1° SEED. 2° SEED. 3° SEED. or (PRODUCTION) (circle one)

VESSEL DESCRIPTION VESSEL SIZE: 28 L TANK CONFIGURATION: SS # OF BAFFLES: 4 # OF IMPELLERS: 2
OF BLADES: 6 BLADE TYPE RUSHTON IMPELLER DIST., BOTTOM: 13.5" . MIDDLE: 8.5" . TOP: NONE .
SPARGER, TYPE: RING DIAMETER: 3.5" . POSITION: CENTER, UNDER BOTTOM IMPELLER INITIALS: RTO

MEDIUM DESIGNATION: Richard's Modified V-8 Broth

INGREDIENT	GMS/LITER	GMS/ 20 L	MATERIAL LOT #	PERFORMED BY:	REVIEWED BY:
DEXTROSE	10	200	Spunkler 031992	RTO	RTO
KNO_3	10	200	Fisher 901060A	RTO	RTO
$CaCO_3$	3	60	Fisher 923342	RTO	RTO
V-8 JUICE	0.2L/L	4 LITERS	Campbells CW33328 3/96	RTO	RTO
SAG-471	0.1 ml/L	2 ml	Van Waters 01402280071291	RTO	RTO
			RTO		
			10 6-94		

BATCHING INSTRUCTIONS

1. ADD 10 LITERS D.I. WATER TO THE FERMENTOR.
2. SLOWLY ADD THE INGREDIENTS IN THE ORDER LISTED ABOVE. MAKING SURE THEY MIX COMPLETELY.
3. ADJUST PH TO — WITH —. PRE-ADJ. PH: NA POST-ADJ PH: — WITH — N/A
4. ADJUST THE VOLUME IN THE FERMENTOR TO 19.5 LITERS WITH D.I. WATER.
5. STERILIZE THE FERMENTOR FOR 30 MINUTES AT 121 °C. TOTAL HEATING TIME = —
6. ADD — LITERS OF — TO THE FERMENTOR ASEPTICALLY FOLLOWING STERILIZATION.
7. ADD STERILIZED ANTIFOAM (SAG 471) AS REQUIRED TO CONTROL FOAMING.

FINAL VOLUME 20 LITERS = BATCH VOLUME 19.5 L + CONDENSATE VOLUME 0 L + INOCULUM VOLUME 0.5 L

INOCULATION

INOCULUM IDENTIFICATION NUMBER: M.t. NAME: Mycoleptodiscus terrestris AGE: 72 H
APPEARANCE: Orange/Red liquid GROWTH: Fair (PCV, CFU, CPM, OD, ETC.)
INOCULUM PH: 7.4 EXPECTED PH: 7-9 PURITY: OK BY TSA + MICRO INITIALS: RTO

OPERATING CONDITIONS — CHANGES DURING RUN:

TEMPERATURE:	25 °C.	NO CHANGES
AERATION:	20 LPM.	NO CHANGES
AGITATION:	300 RPM.	NO CHANGES
BACK PRESSURE:	10 PSI.	—
PH — - — WITH —		—
ADDITIONS, SHOTS, FEEDS, TO BE ADDED.		—
MAINTAIN — % DISSOLVED OXYGEN.		—

COMMENTS/OBSERVATIONS —

PERFORMED BY: Robert T. Donnelly 10-6-94 DATE REVIEWED BY: [signature] 10/12/94 DATE

FERMENTATION DATA

Page 2 of 2

FERMENTOR: 28L-2 LOT NUMBER: R94015 DATE: 10-6-94

BATCH NUMBER: Mt-2 PROJECT NUMBER: 6191 CULTURE: Mt (AS)

POST-STER. pH: 5.9 POST-INOC VOLUME: 20L INITIALS: RTO

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

DATE 1994	TIME	AGE (HRS)	TEMP. (°C)	AIR (LPM)	PRESS. (PSI)	AGIT. (RPM)	INST. pH	MEASURED pH	%DISS. OXYGEN	ASSAY RESULT	COMMENTS: PCV, ADDITIONS, ETC.	OPERATOR
10-6	1500	0	28	20	10	300	—	5.9	—	—	———	RTO
10-7	0700	16	28/25	20	10	300	—	6.3	—	—	———	RTO
10-10	0700	88	25	11/20	19/10	300	-	8.4	—	—	———	RTO
10-11	0700	112	25	18/20	13/10	300	—	8.5	—	—	Changing air filter	RTO
10-12	0700	136	25	17/20	14/10	300	—	8.7	—	—	Harvest ready	RTO
10-12	1000	139	25	20	10	300	—	8.7	—	—	Harvested	RTO

RTO 10-12-94

HARVEST PROCEDURES

HARVEST AGE: 139 Hrs HARVEST pH: 8.7 HARVEST VOLUME: 20L HARVEST ASSAY: NA

HARVEST PURITY: OK BY TSA + MICRO INITIALS: RTO

REQUESTED TREATMENT AT HARVEST: Cent. to collect solids

DELIVER WHOLE BEER, BROTH OR (SOLIDS) (CIRCLE ONE) TO: Bill Hanson YIELD: 1.3Kg

COMMENTS:

PERFORMED BY: Richard T. O'Donnell DATE: 10-12-94

REVIEWED BY: [signature] DATE: 10/12/94

FERMENTATION RECORD AGCHEM/MYCRO Page 1 of ____

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

FERMENTATION Mycoleptodiscus FERMENTOR: 28L-1 DATE: 11-3-94

LOT NUMBER (CUMULATIVE): R94016 PROJECT NUMBER: 5716 BATCH #: Mt-3

CULTURE#: Mt FERMENTATION STAGE: 1° SEED. 2°SEED. 3° SEED. or (PRODUCTION) (circle one)

VESSEL DESCRIPTION VESSEL SIZE: 28 L TANK COMPOSITION: SS # OF BAFFLES: 4 # OF IMPELLERS: 2

OF BLADES: 6 BLADE TYPE RUSHTON IMPELLER DIST., BOTTOM: 13.5" . MIDDLE: 8.5" . TOP: NONE .

SPARGER, TYPE: RING DIAMETER: 3.5" POSITION: CENTER, UNDER BOTTOM IMPELLER INITIALS: RTC

MEDIUM DESIGNATION: RICHARD'S MODIFIED V-8 BROTH

INGREDIENT	GMS/LITER	GMS/ 20 L	MATERIAL LOT #	PERFORMED BY:	REVIEWED BY:
DEXTROSE	10	200	Spindler 131992	RTC	RTC
KNO_3	10	200	Fisher 961060A	RTC	RTC
$CaCO_3$	3	60	Fisher 923342	RTC	RTC
V-8 JUICE	0.2L/L	4 LITERS	Campbells CU332B	RTC	RTC
SAG 471	0.1 ml/L	2 ml	Union Carbide 00K2RN071191	RTC	
		RTC			
		11-3-94			

BATCHING INSTRUCTIONS

1. ADD 10 LITERS D.I. WATER TO THE FERMENTOR.
2. SLOWLY ADD THE INGREDIENTS IN THE ORDER LISTED ABOVE, MAKING SURE THEY MIX COMPLETELY.
3. ADJUST PH TO ____ WITH ____. PRE-ADJ. PH: ____ POST-ADJ PH: ____ WITH ____ KLS
4. ADJUST THE VOLUME IN THE FERMENTOR TO 19.5 LITERS WITH D.I. WATER.
5. STERILIZE THE FERMENTOR FOR 30 MINUTES AT 121 °C. TOTAL HEATING TIME = 2 HRS
6. ADD ____ LITERS OF ____ TO THE FERMENTOR ASEPTICALLY FOLLOWING STERILIZATION.
7. ADD STERILIZED ANTIFOAM (SAG 471) AS REQUIRED TO CONTROL FOAMING.

FINAL VOLUME 20 LITERS = BATCH VOLUME 19.5 L + CONDENSATE VOLUME 0 L + INOCULUM VOLUME 0.5 L

INOCULATION

INOCULUM IDENTIFICATION NUMBER: M.T. NAME: Mycoleptodiscus terrestris AGE: 72 HRS

APPEARANCE: DARK ORANGE SLURRY GROWTH: Very Good (PCV, CFU, CPM, OD, ETC.)

INOCULUM PH: N/A EXPECTED PH: NA PURITY: OK BY TSA + MICRO INITIALS: RTC

OPERATING CONDITIONS — CHANGES DURING RUN:

TEMPERATURE:	25 °C.	NO CHANGES
AERATION:	20 LPM.	NO CHANGES
AGITATION:	300 RPM.	NO CHANGES
BACK PRESSURE:	10 PSI.	
PH ____ - ____ WITH ____		
ADDITIONS, SHOTS, FEEDS, TO BE ADDED.		
MAINTAIN ____ % DISSOLVED OXYGEN.		

COMMENTS/OBSERVATIONS ____

PERFORMED BY: Richard T. O'Donnell 11-3-94 DATE REVIEWED BY: [signature] 12/14/94 DATE

FERMENTATION DATA

Page 2 of 2

FERMENTOR: 28L-1 LOT NUMBER: R94016 DATE: 11-3-94

BATCH NUMBER: Mt-3 PROJECT NUMBER: 6191 CULTURE: Mt (AS)

POST-STER. pH: 6.8 POST-INOC VOLUME: 20L INITIALS: RTC

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

DATE 1994	TIME	AGE (HRS)	TEMP. (°C)	AIR (LPM)	PRESS. (PSI)	AGIT. (RPM)	INST. pH	MEASURED pH	DISS. OXYGEN	ASSAY RESULT	COMMENTS: PCV, ADDITIONS, ETC.	OPERATC
11-3	1400	0	25	20	10	300	—	6.8	—	—		RTC
11-4	0700	17	25	20	10	300	—	6.7	—	—		RTC
11-7	0700	89	25	15/20	15/10	300	—	8.1	—	—		RTC
11-8	0700	113	25	17/20	13/10	300	—	8.5	—	—		RTC
11-8	0900	115	25	20	10	300	—	8.5	—	—	Harvested	RTC

RTC
11-8-94

HARVEST PROCEDURES

HARVEST AGE: 115 HRS HARVEST pH: 8.5 HARVEST VOLUME: 20L HARVEST ASSAY: NA

HARVEST PURITY: OK BY TSA + MICRO INITIALS: RTC

REQUESTED TREATMENT AT HARVEST: Cent for solids

DELIVER WHOLE BEER, BROTH OR (SOLIDS) (CIRCLE ONE) TO: Bill Henson YIELD: 1.2 Kg

COMMENTS:

PERFORMED BY: Richard T. O'Donnell DATE: 11-8-94

REVIEWED BY: [signature] DATE: 12/14/94

FERMENTATION RECORD AGCHEMMICRO Page 1 of _____

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

FERMENTATION MYCOLEPTODISCUS FERMENTOR: 28L-2 DATE: 11-3-94

LOT NUMBER (CUMULATIVE): R9401X7 PROJECT NUMBER: 5716 BATCH #: ML-4

CULTURE: ML. FERMENTATION STAGE: 1° SEED. 1°SEED. 1° SEED. or (PRODUCTION) (circle one)

VESSEL DESCRIPTION VESSEL SIZE: 28 L TANK COMPOSITION: SS # OF BAFFLES: 4 # OF IMPELLERS: 2

OF BLADES: 6 BLADE TYPE RUSHTON IMPELLER DIST.. BOTTOM: 13.5" . MIDDLE: 8.5" . TOP: NONE.

SPARGER, TYPE: RING DIAMETER: 3.5" . POSITION: CENTER, UNDER BOTTOM IMPELLER INITIALS: RTC

MEDIUM DESIGNATION: RICHARD'S MODIFIED V-8 BROTH

INGREDIENT	GRS/LITER	GRS/ 20 L	MATERIAL LOT #	PERFORMED BY:	REVIEWED BY:
DEXTROSE	10	200	Spindletop 031992	RTC	RTC
KNO_3	10	200	Fisher 910460A	RTC	RTC
$CaCO_3$	3	60	Fisher 923342	RTC	RTC
V-8 JUICE	0.2L/L	4 LITERS	Campbell's CU332B	RTC	RTC
SAG 471	0.1 ml/L	2 ml	Union Carbide 0002RN071291	RTC	RTC
		RTC			
		11-3-94			

BATCHING INSTRUCTIONS

1. ADD 10 LITERS D.I. WATER TO THE FERMENTOR.
2. SLOWLY ADD THE INGREDIENTS IN THE ORDER LISTED ABOVE. MAKING SURE THEY MIX COMPLETELY.
3. ADJUST PH TO ____ WITH ____. PRE-ADJ. PH: ____ POST-ADJ PH: ____ WITH ____ MLS
4. ADJUST THE VOLUME IN THE FERMENTOR TO 19.5 LITERS WITH D.I. WATER.
5. STERILIZE THE FERMENTOR FOR 30 MINUTES AT 121 °C. TOTAL HEATING TIME = 2 HRS
6. ADD ____ LITERS OF ____ TO THE FERMENTOR ASEPTICALLY FOLLOWING STERILIZATION.
7. ADD STERILIZED ANTIFOAM (SAG 471) AS REQUIRED TO CONTROL FOAMING.

FINAL VOLUME 20 LITERS = BATCH VOLUME 19.5 L + CONDENSATE VOLUME 0 L + INOCULUM VOLUME 0.5 L

INOCULATION

INOCULUM IDENTIFICATION NUMBER: M.T. NAME: MYCOLEPTODISCUS TERRESTRIS AGE: 72 HRS

APPEARANCE: DARK ORANGE SLURRY GROWTH: Very Good (PCV, CFU, CPM, OD, ETC.)

INOCULUM PH: NA EXPECTED PH: NA PURITY: OK BY TSA+MICRO INITIALS: RTC

OPERATING CONDITIONS — CHANGES DURING RUN:

TEMPERATURE:	25 °C.	NO CHANGES
AERATION:	20 LPM.	NO CHANGES
AGITATION:	300 RPM.	NO CHANGES
BACK PRESSURE:	10 PSI.	
PH ___ - ___ WITH ___		
ADDITIONS, SHOTS, FEEDS, TO BE ADDED.		
MAINTAIN ___ % DISSOLVED OXYGEN.		

COMMENTS/OBSERVATIONS

PERFORMED BY: Richard T. Donald 11-3-94 DATE REVIEWED BY: [signature] 12/16/94 DATE

FERMENTATION DATA

Page 2 of 2

FERMENTOR: 28L-2 LOT NUMBER: R94017 DATE: 11-3-94

BATCH NUMBER: Mt-4 PROJECT NUMBER: 6191 CULTURE: Mt (AS)

POST-STER. pH: 6.2 POST-INOC VOLUME: 20L INITIALS: RTO

Ricerca Inc.
7528 Auburn Road, P.O. Box 1000
Painesville, Ohio 44077-1000

DATE 1994	TIME	AGE (HRS)	TEMP. (°C)	AIR (LPM)	PRESS. (PSI)	AGIT. (RPM)	INST. pH	MEASURED pH	%DISS. OXYGEN	ASSAY RESULT	COMMENTS: PCV, ADDITIONS, ETC.	OPERATOR
11-3	1400	0	25	20	10	300	—	6.2	—	—	—	RTO
11-4	0700	17	25	20	10	300	—	6.2	—	—	—	RTO
11-7	0700	89	25	11/20	19/10	300	—	8.6	-	—	—	RTO
11-8	0700	113	25	15/20	15/10	300	—	8.8	—	—	—	RTO
11-8	1100	117	25	20	10	300	—	8.8	—	—	Harvested	RTO
							RTO					
							11-8-94					

HARVEST PROCEDURES

HARVEST AGE: 117 HRS HARVEST pH: 8.8 HARVEST VOLUME: 20L HARVEST ASSAY: NA

HARVEST PURITY: OK BY TSA - MICRO INITIALS: RTO

REQUESTED TREATMENT AT HARVEST: Cent for solids

DELIVER WHOLE BEER, BROTH OR (SOLIDS) (CIRCLE ONE) TO: Bill Henson YIELD: 1.35kg

COMMENTS:

PERFORMED BY: Richard T. O'Donnell DATE: 11-8-94

REVIEWED BY: [signature] DATE: 12/16/94

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13. ABSTRACT *(Maximum 200 words)*

This report documents development of endemic pathogens as potential inundative biological control agents for *Hydrilla verticillata* and *Myriophyllum spicatum*. Prototype formulations of *Mycoleptodiscus terrestris* were tested on rooted plants of *H. verticillata*. The ability to adhere to hydrilla under submersed conditions was met by each of the formulations tested. For ease of application, the granular formulation was superior to both the suspension concentrate and the two invert emulsions. Maintenance of fungal viability through formulation processes of drying and milling continues to be a formidable problem. Control of a target plant may be enhanced by timing an application that optimizes fungal growth and development with certain weak points in the host. Preliminary results indicate that hydrilla may be more susceptible to pathogen ingress early in a growing season, a period when temperatures are more conducive to fungal growth. Hydrilla plants stressed by an initial application of inoculum are little affected by a second dose applied when plant regrowth commences. The second application of inoculum was shown to yield only an additional 5 percent reduction to the standing biomass of aboveground hydrilla. Efforts continue to find an endemic pathogen of *M. spicatum* that is effective in the field. Thirty pathogens, isolated from milfoil collected from different geographic regions in the United States, were screened for pathogenicity. Isolates that produced disease symptoms in the host during the initial laboratory screening were further evaluated in greenhouse and field tests. Three isolates reduced aboveground milfoil biomass between 95 and 100 percent in greenhouse studies but were ineffective in the field.

14. SUBJECT TERMS
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