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Development of a fungal pathogen for biocontrol of the submersed aquatic macrophyte *Hydrilla verticillata*

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Abstract. The submersed aquatic plant, *Hydrilla verticillata* presents a major challenge for biological control using pathogens. Characteristics which seemingly make hydrilla an ideal target for biocontrol, including perennial growth and the formation of extensive monocultures, are offset by innate problems of a submersed aquatic environment, immediate dilution factors and contact-time in flowing water. Success has been achieved in laboratory and small-scale field trials with a fungal pathogen, *Mycoleptodiscus terrestris*, as a potential inundative agent for management of hydrilla. Above-ground biomass of hydrilla, grown in laboratory aquaria, was reduced by 99% following treatment with a mycelial matrix of the fungus. Similarly, application of the fungus to small, enclosed field-plots resulted in a 63% reduction in above-ground hydrilla biomass four weeks after application. Current research efforts are directed at mycoherbicide formulation. Particularly challenging has been finding compounds which adhere to the submersed plant in flowing water. Experimental formulations which show promise include extruded granules, suspension concentrates, and inverts. Promising results have also been forthcoming combining very low application rates of the herbicide, fluridone, with *M. terrestris*. Integration of chemical and biological control technologies provides the benefits of excellent initial biomass reduction by the fungus combined with long-term hydrilla control provided by fluridone.

Introduction

Hydrilla verticillata (L. fil.) Royle (hydrilla) has been identified as one of the most invasive pests in tropical and subtropical regions (Robson 1976) and has, in recent years, become a problematic species in temperate regions (Steward *et al.* 1984; Steward and Van 1987; Posey *et al.* 1993). Thought to have originated in the warmer areas of Asia (Cook and Luond 1982), today hydrilla is broadly distributed across Asia, extending from Iran and Afghanistan through Pakistan and India to Southeast Asia, then northward into China, Japan, Korea, and the Soviet Union. Documented occurrences indicate that populations are found on all continents with the exception of Antarctica (Cook and Luond 1982; Pieterse 1981).

Since its introduction into Florida in the 1950s (Schmitz *et al.* 1991), hydrilla has greatly expanded its range in the United States of America, radiating throughout the southeast (Haller 1978; Cook and Luond 1982; Langeland and Schiller 1983), moving westward into Louisiana and Texas (Wherry 1974; Guerra 1976), and northward as far as Delaware (Haller 1982). The first documented occurrence of hydrilla on the west coast was in California in 1976 (Sonder 1979). Within the past year (summer 1995), hydrilla was reported for the first time in the state of Washington (Hamel 1995).

The broad distribution of hydrilla arises largely because it can tolerate a wide range of environmental and physical conditions. It can grow and thrive in polluted waters and those rich in nutrients (Cook and Luond 1982). The plant is well adapted to outcompete other aquatic species. Among its attributes are rapid growth, adaptation to very low light-intensities, vegetative reproduction from fragmentation, and development of specialized survival structures (tubers and turions) (Pieterse 1981).

Submersed, nuisance, aquatic plant populations are difficult to control or manage. The perennial growth habit and formation of extensive monocultures, which seemingly make them ideal targets for chemical or biological control measures are often offset by innate problems of the aquatic environment, including immediate dilution factors and contact-time in flowing

water. Efforts for hydrilla management have historically focused on mechanical and chemical technologies. Mechanical control is time-consuming and offers only a short-term solution. High costs of registration and, or, reregistation of herbicides (only six are currently registered in the USA for use in aquatic sites) combined with federal, state, and local regulatory requirements suggest a future limit of their use in aquatic systems. Such restrictions have spurred interest in using biological control, either as an alternative strategy or in an integrated approach for hydrilla management.

The biocontrol agent: *Mycoleptodiscus terrestris*

The development of plant pathogens with potential as biocontrol agents for aquatic weeds is one facet of biocontrol research at the U.S. Army Engineer Waterways Experiment Station. A classical approach was not considered as an option when the pathogen programme against hydrilla began in the 1980s because there were restrictions on importation of exotic pathogens and a quarantine facility was not available at the time. Emphasis was placed instead on inundative biocontrol using endemic pathogens. Today, the biocontrol programme using pathogens has a broad scope and encompasses both endemic and foreign exploration for pathogens of submersed aquatic weeds. The classical approach is now a possibility because the importation of exotic pathogens is allowed and testing can be undertaken at the USDA/ARS quarantine facility located at Fort Detrick, Frederick, MD.

During the first phase of the programme, research emphasis was placed on surveying hydrilla populations in the continental USA for fungal and bacterial isolates and screening them for biocontrol potential (Joye and Cofrancesco 1991). Although this phase no longer constitutes the main focus of the programme, research continues in this area on a limited basis. In 1987, a potential agent was found on hydrilla growing in Lake Houston, Texas (Joye 1990). Initially identified and reported to be *Macrophomina phaseolina* (Tassi) Goid (Joye 1990), it was later confirmed to be a strain of *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (B.C. Sutton, IMI, personal communication). Since that time, numerous isolates of *M. terrestris* have been obtained from hydrilla populations throughout the USA and have become part of our extensive collection of fungal isolates from submersed aquatic plants.

The genus *Mycoleptodiscus* is generally recognized on the host by superficial sporodochia, one cell thick, and by 0-2 septate conidia bearing one apical and sometimes a basal, cellular, unbranched, filiform appendage (Sutton and Alcorn 1990). *Mycoleptodiscus terrestris* spores are characterized by having two cells separated by a median septum and with an asymmetrically-attached apical and basal appendage. Microsclerotia develop readily under a wide range of cultural conditions both on artificial media and within the tissues of the host. Conidial formation is much more sporadic. Conidia seem to develop readily on excised host tissue, infrequently on some solid media, and have yet to be induced to form in broth cultures. Because the fungus fails to sporulate in broth culture, it has been necessary to use a mycelial slurry in all experimentation. The mycelial mat which develops in submerged culture is filtered, rinsed, and ground with sterile water in a blender to produce the slurry for inoculation.

Joye and Paul (1991) examined the histology of infection of hydrilla by *M. terrestris*. They documented events at the cellular level as the fungus attached, penetrated and colonized the host during the first 72 h after inoculation. By 288 h, plants lacked cellular integrity and collapsed. Entry into the host was gained by direct penetration of the fungus through the cell wall. Because there was no sign of bending of host cell-walls, penetration was suggested to be a result of enzymatic activity rather than mechanical pressure.

Laboratory and field testing

Efficacy tests performed under controlled conditions in the greenhouse were extremely promising. A mycelial slurry of *M. terrestris* applied to hydrilla grown in 13.75x150 cm tubes reduced above-ground biomass of treated plants 95-99%, 21 days after inoculation (Joye 1990). The first observable symptom of disease was inter-veinal chlorosis which progressed to a complete loss of leaf colour, followed by plant collapse and disintegration in 10 days. Subsequent experiments with dosage responses indicated that inoculation levels at an effective rate of 100 colony forming units (cfus)/ml (i.e. the cfu rate allowing for dilution in a column of water) resulted in leaf chlorosis and flaccid leaves and stems. When inoculum levels were increased to 400 cfus/ml they were lethal (Shearer 1995).

When experiments were up-scaled from laboratory and greenhouse trials to outdoor tanks and field plots,

the total impact of the fungus on hydrilla lessened, even though the inoculum was applied at the same effective rates. In tank studies, the biomass of hydrilla that was collected one month after inoculation with *M. terrestris* was reduced by 89%, compared to untreated controls. In 1988 and 1989, the biomass in enclosed, hydrilla-inoculated field plots at two weeks after application was reduced by 61.3 and 58% respectively. In more recent field trials, the hydrilla biomass one month after application was reduced by 63% (Shearer 1995).

It appears that as the size of the experimental container or plot increases, dilution of the inoculum and contact time of the fungus with the target plant become increasingly important factors. Within a small container, the fungus applied as a mycelial slurry is confined within a fixed space and the chances of contact with the host are great. In the field, the slurry becomes diluted in the large volume of water to which it is applied. As efficacy testing is up-scaled from small containers in the laboratory to large field-plots, contact time with the target plant may be affected by natural water movements in the submerged plant beds. Such movements have the potential of keeping the inoculum in suspension thus preventing adhesion to the target plant or carrying the inoculum away from the treatment area.

The aforementioned problems and that of sporulation in culture are being addressed through the development of formulations. Allowances for dilution and water movement must be considered in the development of a mycoherbicide destined for use against a submersed aquatic weed, while still maintaining optimum viability and virulence of the fungal component. Extremely important is the amount of contact time the mycoherbicide has with the target plant. It must be of sufficient duration to allow fungal attachment. Several innovative prototype formulations have been developed recently and tested in our laboratories. These include granules, inverts, and emulsions.

From an operational standpoint, granular mycoherbicides are highly desirable because of ease of application. The prototype granule is light and can be applied at the water surface. As it absorbs water, it begins to sink and wafts slowly downwards through the plant mat becoming entangled in the foliage. The main drawback of granule formulations has been loss of fungal viability through drying. In the drying process a reduction of moisture from 44% to about 10% resulted in a logarithmic reduction in cfu counts. With inverts and emulsions, loss of viability became less of a problem because the fungus is not dried, but weight becomes a factor because they are formulated as liquids. Difficulty of application increases because optimum performance of these formulations requires subsurface delivery into the plant mat. Greenhouse experiments in small columns indicated that where contact was made with the plant, typical disease symptoms developed but the highly viscous nature of the formulations prevented even distribution and coverage over the plant surface. While these prototype formulations show excellent promise for overcoming many of the problems associated with developing a mycoherbicide specifically for use in an aquatic environment, many improvements still need to be made in fermentation and formulation before they can be effective and marketable.

An integrated approach

Integrating herbicides with pathogens has been suggested as a viable option for control of submersed vegetation. Sorsa *et al.* (1988) combined the contact herbicide endothall with the fungal pathogen *Colletotrichum* sp. for control of Eurasian watermilfoil. Smit *et al.* (1990) combined the systemic herbicide fluridone with various fungal species for control of coontail (*Ceratophyllum demersum*). Integrating these approaches provided improved efficacy compared with either method on its own.

Using a herbicide such as fluridone can provide long-term control of hydrilla but the long time-lag (weeks to months) between initial treatment and eventual plant death limits its use in aquatic systems. Fluridone acts by inhibiting the biosynthesis of carotenoids which function to absorb light energy to protect chlorophyll molecules from photo-destruction (Bartels and Watson 1978). In contrast, *M. terrestris* can induce leaf chlorosis, defoliation, and stem fragmentation of hydrilla within 14 days of inoculation. The results of field tests suggest that the fungus acts as a contact mycoherbicide and although initial damage may be great, hydrilla has the ability to recover. By combining fluridone with *M. terrestris*, it was thought that the weaknesses inherent in the individual treatments would be offset.

Preliminary results in the laboratory using the integrated approach have shown that complete hydrilla control can be achieved 42 and 60 days after treatment

(DAT) by combining 5 and 12 *ug*/l treatments of fluridone with 200 and 100 cfu/ml treatments of *M. terrestris*, respectively (Netherland and Shearer 1995). A 2 *ug*/l fluridone treatment combined with the two levels of the fungus resulted in complete control of hydrilla by 60 to 94 DAT (Fig. 1). Integrating fluridone with the fungus provided the benefits of excellent initial biomass-reduction exhibited by *M. terrestris* along with long-term hydrilla control provided by fluridone. Combining these treatments greatly reduced fluridone exposure requirements while also reducing the rate of fluridone necessary to provide control of hydrilla. Of significance is the use of both rates of the fungus with a treatment rate of fluridone as low as 2 *ug*/l to produce complete control of hydrilla. These preliminary results demonstrate the potential for combining chemical and biological control agents to improve efficacy, thereby reducing reliance on chemicals through lower dosage rates or less frequent applications.

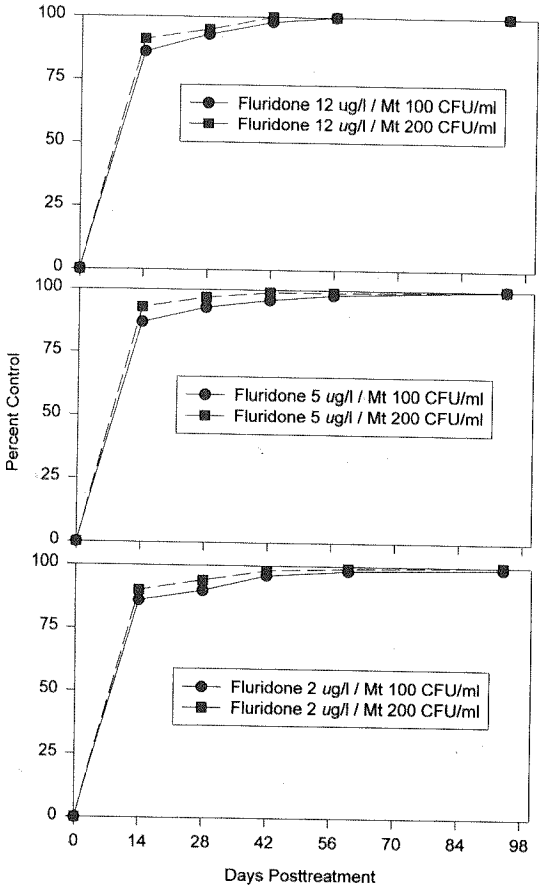

Fig. 1. Percentage control of hydrilla measured as the reduction of shoot biomass following an integrated fluridone/*Mycoleptodiscus terrestris* treatment at several rates. Each data point represents the average of three replicates.

Future efforts in the *M. terrestris*/hydrilla biocontrol system will be directed at host-specificity studies, fermentation and formulation improvement, sporulation enhancement, and a continuation of studies to develop an integrated approach using biocontrol and chemical technologies.

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