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Screening of biological control pathogens isolated from Eurasian watermilfoil

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INTRODUCTION

Surveys to find potential pathogen biological control agents for Eurasian watermilfoil (*Myriophyllum spicatum* L.), hereafter called milfoil, were begun in the United States in the late 1970s (Andrews 1980). Two pathogens, *Fusarium sporotrichioides* Sherbakoff and a *Trichothecium* sp. isolated from milfoil collected in University Bay, Lake Mendota, Wisconsin, caused noticeable damage to milfoil in laboratory tests. Although *F. sporotrichioides* induced the development of localized necrotic stem lesions in milfoil, it was not highly pathogenic (Andrews and Hecht 1981), making it a poor candidate for biological control. The *Trichothecium* species was later determined to be *Acremonium curvulum* W. Gams (Andrews et al. 1981). The weak pathogen also was identified as an endophyte and could be isolated from healthy milfoil plants. When *A. curvulum* was inoculated onto endophyte-infected plants, the plants usually died; however, when it was inoculated onto endophyte-free plants it was only mildly pathogenic and the plants usually recovered. The inconsistent control made it a poor candidate for further development. Another fungus, *Colletotrichum gloeosporioides* (Penz.) Penzig & Saccardo, was tested in J. H. Andrews' lab in the 1980s, and it too demonstrated some promise as a biological control agent (Smith et al. 1989). Unfortunately, when milfoil plants were grown in lake water and a carbonate-buffered artificial medium similar to that encountered in the field, performance was poor and further testing was suspended.

Researchers at the University of Massachusetts at Amherst conducted surveys in Massachusetts and Alabama in the 1980s for pathogens of milfoil. A cellulolytic fungus, *Mycoleptodiscus terrestris* (Gerdeman) Ostazeski, was isolated and found to be efficacious on plants in both laboratory and field studies (Gunner et al. 1988, 1990). It was formulated into the bioherbicide Aqua-Fyte® (EcoScience Laboratories, Worchester, MA) and registered with the Environmental Protection Agency in the early 1990s. The original formulation was a round calcium alginate pellet approximately 3 mm in diameter that did not adhere well to plant material. Consequently, the design was modified to produce short strings (~15 mm length by 2 mm diam.), and the new formulation was tested in plots in a pond located on the Tennessee Valley Authority Murphy Hill field station adjacent to Guntersville Reservoir, Guntersville, Alabama (Shearer 1995). The string formulation provided better attachment to plants but was ineffective in reducing above ground biomass, and EcoScience discontinued further development of the bioherbicide after the failed field test.

Surveys for pathogens of milfoil were also undertaken by the US Army Corps of Engineers (USACE) in the 1980s (Zattau 1988). In total, 462 bacterial and 330 fungal isolates were retrieved from 213 samples collected from 50 milfoil-infested water bodies in 10 states. The isolates were bioassayed for presence of lytic enzymes. Based on the bioassays, 14 bacterial and 22 fungal isolates were selected for further evaluation on milfoil. Although several fungal isolates were determined to be candidates for biological control, none were ever commercially developed and the cultures were subsequently lost. All bacterial isolates were deemed poor candidates and were not studied further.

Although EcoScience discontinued bioherbicide development of *M. terrestris*, USACE continued to examine the potential of an *M. terrestris* isolate obtained from milfoil collected in Guntersville Reservoir by combining it with herbicides in an integrated approach for milfoil control. Integrated treatments combining *M. terrestris* with fluridone significantly reduced milfoil growth 84 d after treatment compared with untreated controls (Nelson and Shearer 2002). Concentrations as low as 5 µg active ingredient (a.i.) L^{-1} fluridone applied simultaneously with 100 colony-forming units per milliliter *M. terrestris* reduced milfoil biomass by 92%, whereas either product applied alone at these rates was ineffective in reducing plant growth (Nelson and Shearer 2002). Similarly, combining 2,4-D with *M. terrestris* reduced shoot biomass of milfoil more effectively than either agent applied alone (Nelson and Shearer 2005). Low doses of the herbicide and pathogen reduced root biomass by 85%, minimizing the potential for plant regrowth following treatment. Additionally, combining *M. terrestris* with triclopyr provided enhanced control of milfoil at low rates of both agents (Nelson and Shearer 2008).

Since the surveys were undertaken in the 1970s and 1980s, milfoil has expanded its range to all 48 contiguous states (US Department of the Interior 2005) and Alaska, being absent only from Hawaii (USDA, NRCS 2010). Presently there are no classical insect or pathogen biological control agents available for managing this invasive submersed aquatic weed. Three natural enemies studied in the United States are *Acentria ephemerella* Denis and Schiffermüller, a naturalized pyralid moth; *Euhrychiopsis lecontii* Dietz, a native weevil; and *Cricoptopus myriophylii* Oliver, a midge that was probably an accidental introduction (Johnson and Blossey 2004). At present, only *E. lecontei* is in commercial production by Enviroscience Inc., Stow, Ohio. Although declines in some lakes

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have been attributed to the weevil (Creed and Sheldon 1995, Sheldon and Creed 1995, Sheldon 1997, Newman and Biesboer 2000), its performance is not always consistent and more research is needed (Newman and Biesboer 2000). Grass carp are only used occasionally because milfoil is not a preferred plant species (Sanders et al. 1991). Additional agents are needed to manage this rapidly expanding invasive species; therefore, 30 years after the first surveys were undertaken, additional surveys were initiated in 2009 to search for new pathogen biological control agents for milfoil. This study documents the results of those surveys.

MATERIALS AND METHODS

During summer 2009, surveys were conducted in various geographical regions of the United States to collect milfoil samples for the purpose of isolating potential pathogenic biological control agents. In total, 53 milfoil samples were collected from sites in 12 states (OR, WA, IA, NE, MO, MN, AL, VA, MA, NH, VT, and NY) and shipped overnight in refrigerated coolers to the biocontrol laboratory at the US Army Engineer Research and Development Center (USAERDC), Vicksburg, Mississippi. Upon arrival, the samples were thoroughly washed in running water to remove any soil or debris attached to stems and leaves. The samples were wrapped in moist paper toweling, placed in plastic bags, and kept at 4 C until they could be processed.

The samples were processed by dilution plating. A 10 g subsample of stem and leaf tissue was surface sterilized in a 3.5% sodium hypochlorite solution for 1 min and rinsed in deionized water for 1 min. The subsample was blotted dry with sterile paper towels then added to 100 mL of sterile water and macerated in a blender for 30 s, providing a dilution factor of 1/10. The resulting slurry was further diluted to concentrations of 1/100 and 1/50. All dilutions were plated in 1 mL aliquots onto Martin's agar (1 L H_2O; 20 g agar, 0.5 g KH_2PO_4; 0.5 g $MgSO_4 \cdot 7\ H_2O$; 0.5 g peptone; 10 g dextrose, 0.5 g yeast extract; 0.05 g rose Bengal; 0.03 g streptomycin sulfate) plates (3 plates per dilution concentration). The plates were incubated in the dark at 25 C for 1 week. Small pieces (~1 by 1 mm) were cut from the leading edge of filamentous fungal colonies on the plates and transferred to Potato Dextrose Agar (PDA; Difco Inc., Detroit, MI) slants, test tubes placed at an angle during cooling to give a large slanted surface for inoculation. After 7 to10 d, the slants from each of the geographic regions were sorted together and enumerated into morphospecies based on gross colony morphology and color. The cultures were stored at 4 C until they could be plated for identification. Each morphological "species" was plated onto Potato Carrot Agar (PCA; Dinghra and Sinclair 1995) and PDA and incubated at 25 C under a grow light for 1 to 3 weeks to induce sporulation. Both agars are important for isolate identification because characteristic colors and growth patterns develop on PDA, and colonies readily produce asexual and/or sexual spores on PCA. Those cultures that sporulated were identified to genus and species when possible. Those that did not sporulate were placed in categories of moniliaceous (hyaline hyphae) or dematiaceous (dark hyphae) Ascomycetes.

In total, 222 fungi strains were identified and tested for pathogenicity on milfoil. Fungi were selected for screening if they were in a genus with documented pathogenic species (e.g., *Fusarium*, *Phoma*, *Colletotrichum*, *Drechslera*, and *Mycoleptodiscus*). Those fungi recognized to be secondary invaders or saprophytes were not tested. Between 8 and 10 isolates were plated each week onto PDA and allowed to grow 8 to 10 d at 25 C. Approximately one-half the culture on the plate was cut into small pieces (1 by 1 mm) and added to a 250 mL baffled flask containing 100 mL of Richards's V-8 juice broth (10 g glucose; 10 g KNO_3; 3 g $CaCO_3$; 200 mL V-8 juice (Campbells, Camden NJ); 800 mL H_2O). The flasks were placed on a platform shaker (New Brunswick, Edison, NJ) set at 300 rpm. Flasks were swirled daily to prevent fungal build-up along the sides of the flasks. After 7 d, the flasks were examined for contamination and the culture medium characterized by color, amount of mycelium produced (sparse to abundant), presence or absence of microsclerotia (melanized survival structures), chlamydospores, and conidia (thin-walled asexual spores). Contents of the flask were ground in a blender for 30 s to homogenize the culture. The resulting slurry was pipetted in 1 mL aliquots into 250 mL Erlenmeyer flasks containing 100 mL deinonized water and a milfoil apical shoot 15 cm in length. Each treatment was replicated 5 times. The flasks were incubated in a growth chamber (Conviron, Pembina, ND) at 25 C under a 12/12 light/dark photoperiod for 14 d. The milfoil shoots were visibly rated for presence or absence of disease on a rating scale of 0 to 4 (0 = no disease, tissues green and healthy; 1 = slight chlorosis; 2 = general overall chlorosis; 3 = tissues discolored and stems beginning to fragment; 4 = total discoloration and tissues collapsed, no possibility of regrowth).

RESULTS AND DISCUSSION

The number of milfoil samples received from surveys in different geographic regions of the United States in 2009 included: Northwest (15), Midwest (11), Southeast (3), and Northeast (24) (Figure 1). Fewer samples were collected in the Southeast primarily due to time constraints. In total, 885 fungal isolates were obtained from milfoil tissues in the 53 samples. After sorting the isolates by color and gross morphology, 457 strains of fungi were enumerated. Of these, 20% could not be identified because they failed to sporulate on PCA or PDA. Roughly 90% of the unidentified species were either dematiaceous or moniliaceous Ascomycota. Once identifications were made, 7 genera (*Acremonium*, *Alternaria*, *Cylindrocarpon*, *Mycoleptodiscus*, *Phoma*, *Plectosphaerella*, and *Trichoderma*) were found on milfoil plants in each of the geographic regions. They also comprised 46% of the total isolates obtained during the study.

In total, 42 fungal strains, 33 of which were *M. terrestris*, impacted milfoil at a mean disease value of 2 or greater. The other strains were an unknown Coelomycete, *Myrothecium roridum*, *Drechslera* sp., an arthrosporic species, *Calcarisporium* sp., *Colletotrichum* sp., and 3 *Phoma* spp. Strains receiving a mean disease rating of 2 would not be considered for further testing because general chlorosis does not cause sufficient plant damage for them to be considered good biological control agents. Another 33 strains impacted milfoil at a

Figure 1. Sites surveyed for Eurasian watermilfoil pathogens in 2009. Coordinates were not available for one site in the Northwest on the Columbia River and for Lake George in the Northeast.

mean disease value of 3 or greater and would be considered for further testing. Only 5 strains, 4 *M. terrestris* and one *M. roridum*, induced mean disease ratings of 4. These isolates should be tested further as candidates for milfoil management.

Pathogenicity of the strains varied. For example, *M. terrestris* in most screening tests induced a mean disease rating greater than 3; however, a few strains caused little to no damage. These strains were strictly hyphal and failed to produce microsclerotia in culture. The strain currently being used for mycoherbicide development for hydrilla biocontrol produces abundant microsclerotia in agar and broth culture (Shearer and Jackson 2006). There also was variability in pathogenicity of strains of *Myrothecium*, *Colletotrichum*, *Drechslera*, and *Phoma*. Whereas a few species caused significant damage, most caused little to no damage to milfoil sprigs.

Two fungi, *A. curvulum* and *C. gleoesporioides*, that had been studied in Wisconsin as potential pathogen agents for milfoil also were isolated from milfoil during the 2009 surveys. In the Wisconsin studies, the weak pathogen, *A. curvulum*, was found to also occur as an endophyte in milfoil tissues (Andrews et al. 1981). When the pathogen was inoculated onto endophyte-infected milfoil plants, the plants usually died, but when inoculated onto endophyte-free plants, *A. curvulum* was only mildly pathogenic and the plants survived. During the present study, a single isolate of *A. curvulum* was obtained from milfoil collected in Vermont. When screened against milfoil in the USAERDC biocontrol laboratory, it received a disease rating of zero. It is interesting to note that *A. curvulum* did not occur as an endophyte in the milfoil used during the screening.

Six isolates of *C. gleoesporioides* were obtained from milfoil during the 2009 surveys. When screened against milfoil the mean disease rating was only 0.05. Although the Wisconsin isolate was more pathogenic than those isolated during the present study, performance was inconsistent and further studies were curtailed by the Wisconsin researchers (Smith et al. 1989). An assessment of fungal pathogens found on milfoil in Europe documented that *C. gleoesporioides* provided good control, but lack of host specificity also curtailed its further development into a classical biological control agent (Harley and Evans 1997).

More than three-fourths of the species screened for pathogenicity to milfoil produced microsclerotia. In total, 29 of the 33 strains (or 87.9%) that induced a disease rating of ≥3 produced microsclerotia. This has important implications for bioherbicide development because the USAERDC and the United States Department of Agriculture National Center for Agricultural Utilization Research hold a joint patent on production of fungi for use on aquatic plants. Any fungus that produces microsclerotia in broth culture and can be used to manage an aquatic invasive species is covered by the

patent. In contrast, production of conidia in broth culture was not an indicator that a fungal strain would be a potential pathogen biological control agent. None of the 33 strains that induced a disease rating of 3 or greater on milfoil produced conidia in broth culture.

Additional surveys are planned for summer 2010 and will include searching for new milfoil and hydrilla biocontrol agents. Strains that caused high disease ratings on milfoil in the present study will also be evaluated on rooted milfoil in 55 L aquariums in growth chamber studies. The newly isolated pathogenic strains have excellent potential for development into bioherbicides for milfoil management.

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