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Effect of Dried *Mycoleptodiscus terrestris* Inoculums on Fluridone-Susceptible and Fluridone-Resistant Hydrilla Biotypes

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PURPOSE: This technical note describes an aquarium study conducted to determine the efficacy of dried *Mycoleptodiscus terrestris* (Gerd.) Ostazeski against fluridone-susceptible and fluridone-resistant hydrilla biotypes.

INTRODUCTION: *Hydrilla verticillata* (L.f.) Royle, a member of the Hydrocharitaceae, was introduced into Florida in the 1950's (Schmitz et al. 1991). The introduction was the dioecious biotype with only gynoecious plants making up the original infestation. Today hydrilla has invaded at least 21 states either as dioecious or monoecious biotypes or both (Richardson 2008). Called "the perfect aquatic weed" by Langeland (1996), the plant has several characteristics that make it an excellent competitor in freshwater systems. It has low light and carbon dioxide compensation points (Van et al. 1976) and it reproduces by several means including fragmentation, stolons, and turions that develop in leaf axils or subterraneously from rhizomes (Langeland 1996). Seeds have been reported in monoecious hydrilla populations (Langeland and Smith 1984) but are in all likelihood not a major reproductive mechanism.

For years fluridone was the chemical of choice for hydrilla management. It was thought that because the dioecious biotype reproduced only vegetatively, herbicide resistance would not become a problem. However, in the late 1990's, a differential response to fluridone was noted in some hydrilla-infested lakes in Florida. Studies by Michel et al. (2004) of 200 water bodies in Florida revealed that at least 20 of them had hydrilla phenotypes with two- to six-fold higher fluridone resistance. Further genetic research found three independent herbicide-resistant biotypes that arose from the selection of somatic mutations at the arginine 304 codon of the enzyme phytoene desaturase (pds) (Michel et al. 2004). Fluridone is an inhibitor of the enzyme. Following cloning of the gene for pds from both herbicide-susceptible and herbicide-resistant hydrilla biotypes, it was found that the three pds variants had specific activities similar to the wild type but were two to five times less sensitive to fluridone (Michel et al. 2004).

Historically, methods utilized for hydrilla control have included mechanical, chemical, and biological technologies. Mechanical control is expensive, costing in excess of $1000 per acre, and poses some logistical problems in disposing of the harvested biomass (Langeland 1996). Control is often of short duration and several harvests are necessary per year. Alternative chemicals to fluridone for hydrilla management include copper products, diquat, endothall, imazamox, and penoxsulam. Similar to fluridone, the enzyme acetolactate synthase (ALS) inhibitors, imazamox and penoxsulam, require long contact times (up to 90 days) to achieve hydrilla control. All of the other alternative herbicides are fast acting and provide more rapid kill than penoxsulam, imazamox, or fluridone.

Both triploid grass carp and insects have been released as biological control agents for hydrilla. Although success has been achieved with grass carp in some systems (Kirk et al. 2000), Richardson (2008) advised that additional research is needed to better predict plant and fish response to stocking rates and to evaluate the potential of using grass carp in integrated weed management. Four insects, *Hydrellia pakistanae*, *Hydrellia balciunasi* (two leaf-mining flies), *Bagous affinis*, and *Bagous hydrillae* (two weevils) have been approved for release in the United States; however, only the leaf-mining flies have become established (Balciunas et al. 2002). While both leaf mining flies have become established, *H. pakistanae* is found with greater frequency than *H. balciunasi* throughout their established range (Grodowitz 2003). Several years following release of *H. pakistanae*, plant declines were noted at Coleto Creek Reservoir, Texas; Lake Seminole, Florida; and Sheldon Reservoir, Texas (Grodowitz 2003).

Surveys to look for indigenous pathogens that might have potential for hydrilla management began in the late 1980's in the southeastern United States. During the 1987 survey, a potential candidate was found on hydrilla in Lake Houston, Texas (Joye and Cofrancesco 1991). Initially identified as *Macrophomina phaseolina*, it was later determined to be *Mycoleptodiscus terrestris* (Shearer 1996). Testing of the isolate in the greenhouse and in small field plots at Sheldon Reservoir, Texas, determined that the isolate could significantly reduce hydrilla biomass compared to untreated controls (Joye 1990). In 1991, field collections from Sheldon Reservoir yielded a more aggressive *M. terrestris* isolate and this strain has been the subject of further investigations for use alone or in conjunction with herbicides for hydrilla management (Shearer and Jackson 2006; Shearer and Nelson 2002; Shearer 1998; Nelson et al. 1998; Netherland and Shearer 1996).

In 2000, the U.S. Army Engineer Research and Development Center (ERDC) in cooperation with the United States Department of Agriculture, National Center for Agricultural Utilization Research (USDA/NCAUR) crop bioprotection working group, developed a new method for growing *M. terrestris* in broth culture that resulted in a U.S. patent #6,569,807 (Shearer and Jackson 2003). The broth medium could be used alone as inoculum or the melanized survival propagules (microsclerotia) that developed in the broth medium could be harvested and dried down to a moisture content between 5 and 10 percent (Shearer and Jackson 2006). The broth inoculum must be used within a few days because *M. terrestris* rapidly loses viability. The dried granules could be vacuum packed and stored under refrigeration at 4 ºC until needed. They survive without losing viability or virulence for as long as one year.[1]

The Sheldon Reservoir strain of *M. terrestris* has proven efficacious on fluridone-susceptible hydrilla in greenhouse and mesocosm tests (Shearer 1996, 1998; Netherland and Shearer 1996; Nelson et al. 1998). In 2001, both fluridone-susceptible and fluridone-resistant hydrilla were challenged by liquid inoculum of *M. terrestris* that was grown in broth culture using cotton seed meal or corn steep liquor powder as a nitrogen source. Compared to the untreated controls, shoot biomass of both fluridone-susceptible and fluridone-resistant hydrilla was significantly reduced using liquid inoculum applied at rates of 0.1 ml L^{-1} and 0.2 ml L^{-1} (Shearer and Jackson 2006). Inoculum produced in a medium with corn steep liquor powder reduced hydrilla shoot biomass of both biotypes by approximately 99 percent 4 weeks after inoculation. It appears that genetic changes

[1] Personal observation, Judy Shearer.

that led to fluridone resistance do not seem to affect susceptibility to the pathogen when it is applied as a liquid inoculum.

The goal of the cooperative work between NCAUR and ERDC has been to develop *M. terrestris* into a bioherbicide. Toward that end, methods have been refined so that *M. terrestris* is grown in broth culture and the microsclerotia are harvested by dewatering the broth medium. The harvested microsclerotia are ground in a specialized mill, dried at room temperature overnight, vacuum packed, and stored at 4 ºC until needed. To determine if pathogen viability or virulence might be compromised by the new processes, fluridone-susceptible and fluridone-resistant hydrilla were challenged with granular *M. terrestris* to determine if there were differences in efficacy depending on the hydrilla biotype.

MATERIALS AND METHODS: Microsclerotia were produced at NCAUR in a 100-L fermentation unit in a liquid culture medium developed by Shearer and Jackson (2006). The microsclerotia were harvested with a vacuum filtration drum that removed the liquid spent medium and loaded the propagules onto diatomaceous earth (HYFLO, Celite Corp., Lompoc, CA). As the drum turned, a cutting blade sliced the microsclerotia/diatomaceous earth mixture into thin layers. The layers were cut into strips approximately 6 cm wide by 25 cm long and fed into a milling apparatus (Quadro, Ontario, Canada). As the strips entered the mill, a rotating impeller forced them onto a screen surface where they were granulated and all granules $\leq 1397\mu$ tangentially passed through the screen openings and were collected on large aluminum trays. The granules were distributed into a thin layer and allowed to air dry to moisture contents of 31.4, 19.6, 14.4, and 5.6 percent. The granules were then vacuum packed into plastic bags, shipped overnight to ERDC in a cooled container, and stored at 4 °C until needed.

The number of microsclerotia per milliliter of broth medium was determined at NCAUR by placing 100 µl of a 10^{-1} dilution of culture broth on a glass slide and overlaying it with a coverslip. All well-formed microsclerotia on the slide were counted. During sampling, microsclerotium suspensions were constantly vortexed to ensure homogeneity. To enumerate spore production (sporogenic germination), water agar plates were sprinkled with 25 mg of dried microsclerotia (three plates per treatment) and incubated in a growth chamber (Conviron, Pembina, ND) set to a 14/10 light:dark photoperiod at 25 ºC. After 7 days, the plates were removed from the growth chamber and flooded with 5 ml of sterile water. The spores were dislodged from the microsclerotia with a sterile loop and counted using a hemacytometer. Total number of spores per gram *M. terrestris* microsclerotia was determined by multiplying the spore count by 5 (the amount of water used to flood the water agar plate) and dividing the product by the weight of the sample (0.25 g).

Plant material for aquarium studies was obtained from Lake Okahumpka, FL (fluridone-resistant hydrilla) and from the U.S. Army Corps of Engineers Lewisville Aquatic Ecosystem Research Facility (LAERF), Lewisville, TX (fluridone-susceptible hydrilla). Both hydrilla biotypes were maintained in 1200-L culture tanks in the aquatic plant greenhouse at ERDC until needed for aquarium studies.

Thirty 55-L aquaria (0.9 m tall x 0.09 m^2) were filled with a water-based culture solution (Smart and Barko 1985). Lake sediment collected from Brown's Lake at ERDC was amended with ammonium chloride (0.5 g L^{-1}) and Esmigran (1.7 g l^{-1}). Four plastic cups (0.95 L) filled three-fourths with amended lake sediment were planted with five 20-cm apical cuttings from dioecious hydrilla,

overlain with silica sand to prevent sediment resuspension and algal growth, and placed in each aquarium. Half of the aquaria were planted with fluridone-susceptible hydrilla and half were planted with fluridone-resistant hydrilla. The plants were allowed to grow for 28 days, by which time they had reached the water surface.

Inoculum for the experiment was weighed out in 2-g aliquots into plastic weigh boats. The amount of active ingredient (*M. terrestris*) in each aliquot was approximately 1.4, 1.6, 1.7, and 1.9 g taking into consideration moisture contents of 31.4, 19.6, 14.4, and 5.6 percent, respectively, in the four dry samples. The dry inoculum was sprinkled evenly over the water surface of the aquariums and allowed to naturally dissipate onto plant surfaces. The inoculum level following dilution in the water column was 0.04 g l^{-1}. Each treatment was replicated three times. After 28 days, hydrilla shoot biomass was harvested, dried for 4 days at 60 °C to a constant weight, and dry weight was recorded.

Analysis of variance (ANOVA) (Statistica, StatSoft, Tulsa, OK) was used for statistical treatment of data. Mean separations were accomplished using Tukey's Honest Significant Difference (HSD) test. Test of significance was conducted at $P \leq 0.05$.

RESULTS AND DISCUSSION: The broth medium used in this study contained micronutrients (Shearer and Jackson 2006), 3 percent corn steep liquor powder as the nitrogen source, and 6 percent glucose as the carbon source. The number of microsclerotia that developed in the broth medium after a 4-day fermentation was 3.9 x 10^2 L^{-1}. The microsclerotial count was very similar to that found after an 8-day fermentation (Shearer and Jackson 2006) suggesting that microsclerotia numbers do not increase over time. Most microsclerotia are formed by day 4 but they do not become fully melanized until day 8 at which time the hyphal halo no longer exists (Figure 1).

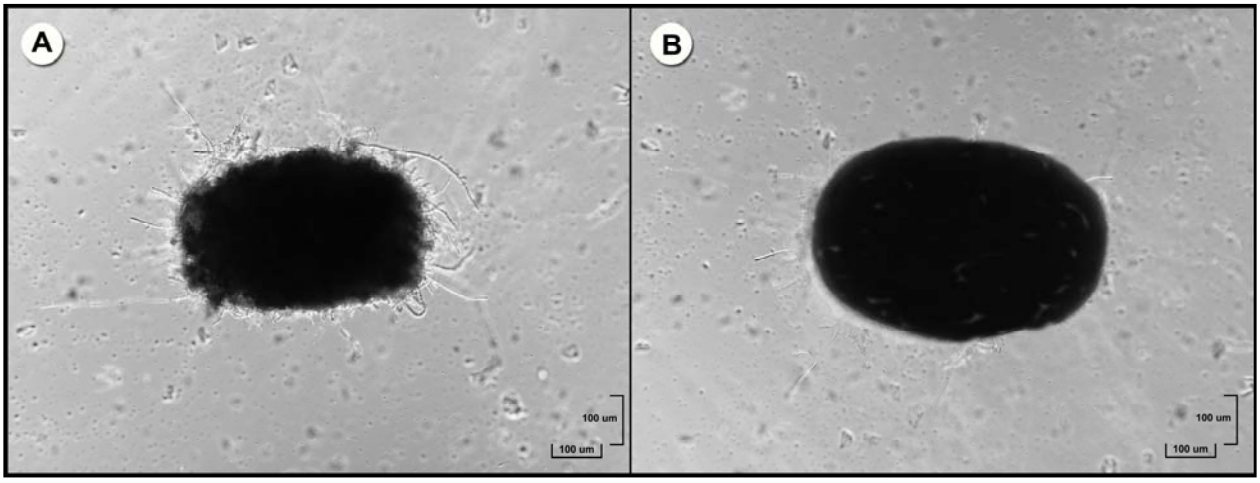

Figure 1. Microsclerotia from a 4-day fermentation (A), and from an 8-day fermentation (B). Note how the hyphal halo is lost as the microsclerotium ages.

Following sprinkling onto water agar plates, spores begin to form over the surface of the microsclerotia by day 3 and a new crop of spores is produced daily over the next several days (Shearer 2007). Although the number of spores g^{-1} of dried *M. terrestris* varied between samples, the means were not statistically different ($P = 0.177$) (Figure 2). The amount of active ingredient in the samples

was not a factor in spore production, since the same amount of dried material (25 mg) was sprinkled onto each plate.

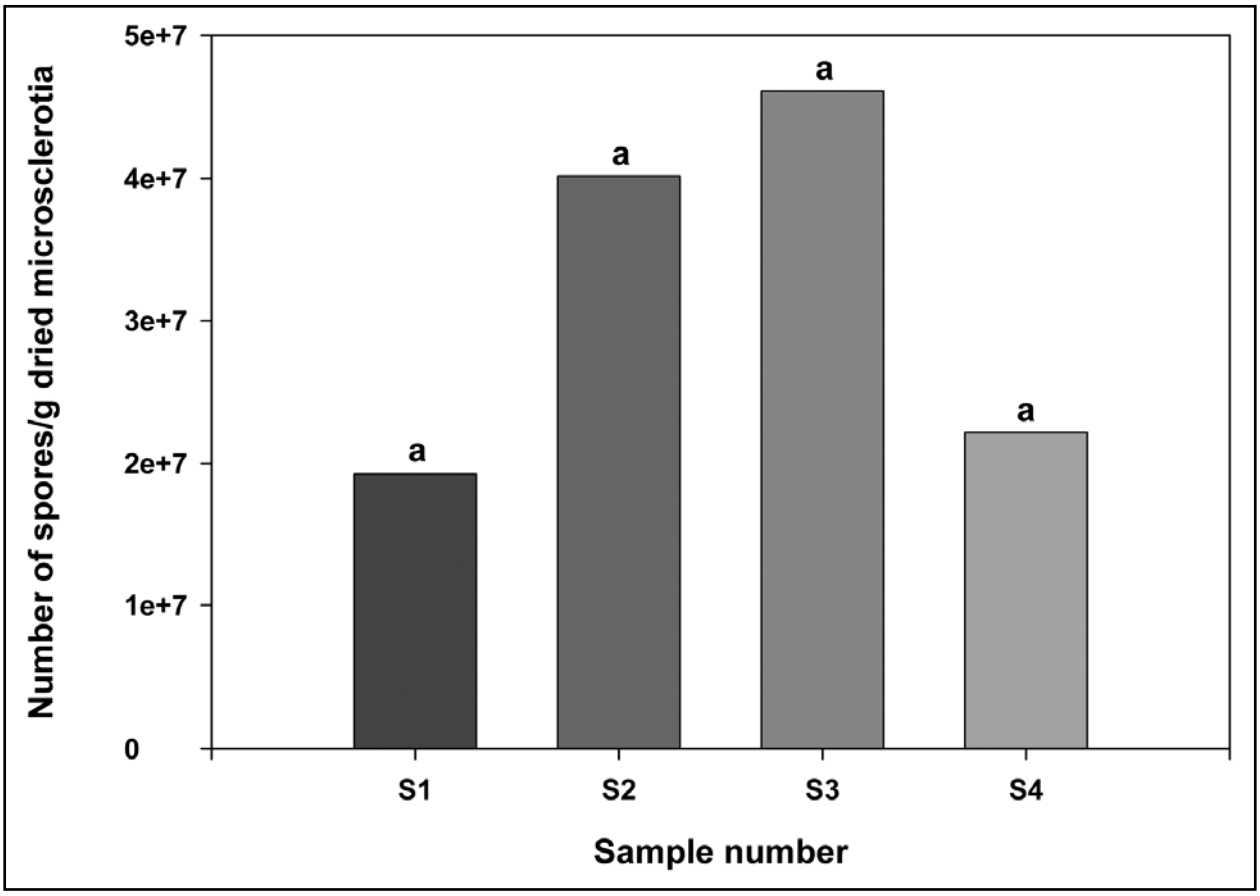

Figure 2. Number of spores g^{-1} dried *M. terrestris* microsclerotia from four samples having differing moisture contents. Bars sharing the same letter do not differ significantly from each other according to Tukey's Honest Significant Difference Test (HSD).

Compared to the untreated control, shoot biomass of both fluridone-susceptible ($F = 51.5$, $P = 0.0000$) and fluridone-resistant hydrilla ($F = 7.21$, $P = 0.0092$) was reduced significantly by application of *M. terrestris* dried inoculum from a 4-day-old fermentation (Figure 3). Genetic changes that led to fluridone resistance (Michel et al. 2004) do not seem to affect susceptibility to dried *M. terrestris*. Here, as in a previous study in which a liquid inoculum was applied (Shearer and Jackson 2006), the treatment with the highest active ingredient performed the best. This treatment level reduced biomass by 88 percent and 100 percent for fluridone-susceptible and fluridone-resistant biotypes, respectively. All other treatments reduced biomass by less than 85 percent. Biomass reductions of less than 85 percent are probably not considered acceptable for management of hydrilla.

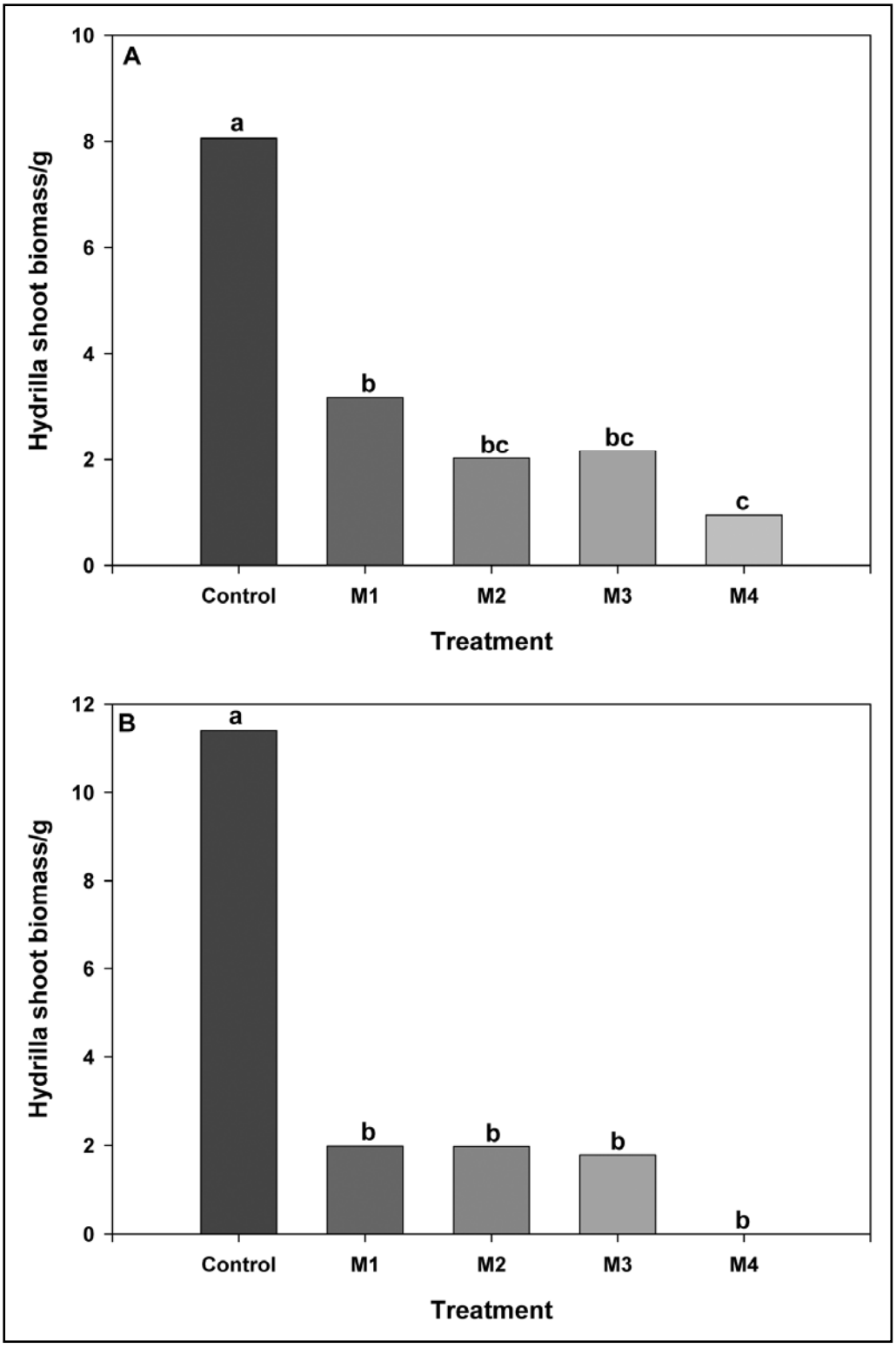

Figure 3. Hydrilla shoot biomass of fluridone-susceptible (A), and fluridone-resistant (B) biotypes 28 days after treatment with dried *M. terrestris* granules. Moisture content in the dried samples was 31.4, 19.6, 14.4, and 5.6 for M1-M4, respectively. Bars sharing the same letter do not differ significantly from each other according to Tukey's Honest Significant Difference Test (HSD).

There are advantages and disadvantages of using liquid versus dry inoculum. The liquid inoculum dissipates faster than the dry and seems to adhere better to plant surfaces. Since *M. terrestris* is a contact pathogen, the more points of contact, the better the chance of infection. The major drawback to liquid inoculum is its minimal shelf life, lasting only a few days before it loses viability. The dry inoculum tends to float on the water surface, which would be a great disadvantage in a field application. It could be rewetted before application and this would alleviate the problem. Granular inoculum that is dried to a moisture content between 5 and 10 percent offers storage stability for up to 7 months without compromising viability or virulence (Shearer and Nelson 2009).

FUTURE WORK: Future research will focus on formulation of the fungus to provide better contact of the fungus with plant surfaces so inoculation rates can be considerably reduced. With completion of small ponds at the ERDC Wetlands and Aquatic Ecosystem Research Center, outdoor testing of dried material on both fluridone-susceptible and fluridone-resistant hydrilla can be undertaken on a larger scale in preparation for field testing.

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