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Effect of dried *Mycoleptodiscus terrestris* inoculums on dioecious hydrilla

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PURPOSE: This technical note describes the results of a growth chamber study and a pond study to evaluate the effectiveness of the fungal pathogen, *Mycoleptodiscus terrestris*, for managing the nuisance submersed plant hydrilla.

INTRODUCTION: A dioecious biotype of *Hydrilla verticillata* (L.f.) Royle (hydrilla) was introduced into Florida in the 1950's and is now found in lakes, ponds, reservoirs, rivers, and canals across the southern United States (Langeland 1996, Schmitz et al. 1991) and in California. The plant is an excellent competitor in aquatic habitats due to its ability to photosynthesize at low light levels, to tolerate diverse environmental conditions, and to produce several survival propagules (Holm et al. 1997). Plant infestations can impede navigation, clog drainage or irrigation canals, affect water intake systems, interfere with recreational activities, and disrupt wildlife habitats. Hydrilla is primarily managed using herbicide applications and to some extent by using specialized harvesting equipment collectively known as mechanical control. The recent appearance of herbicide resistance in some populations of hydrilla combined with the public perception that herbicides are environmental "toxins" has heightened interest in alternative management options including biological-based methods (Richardson 2008).

One alternative management technique being considered for hydrilla is inundation biocontrol with an indigenous pathogen. This approach to weed control is based on the application of infective propagules of a plant pathogen in sufficient numbers to infect and kill the target weed (Harley and Forno 1992, Eilenberg et al. 2001). This method resembles the use of an herbicide in that the biocontrol agent is applied when needed and must contact and kill the host weed (Auld and Morin 1995). A major requirement for the success of such an approach is the use of a virulent pathogen that consistently infects and kills the pest host under field conditions (Jackson 1997; Charudattan 2001).

A survey of hydrilla populations in the southern United States in the 1980's resulted in the isolation of an indigenous fungal pathogen that was capable of infecting and significantly reducing hydrilla biomass in greenhouse and field trials (Joye 1990; Joye and Cofrancesco 1991). The pathogen was originally reported as *Macrophomina phaseolina* (Tassi) Goid but later determined to be *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (Mt) (Shearer 1993). Examination of the host/pathogen relationship at the histological level showed that Mt attached and directly penetrated cell walls of hydrilla (Joye and Paul 1991). Within 8 days, hydrilla tissues were completely colonized by the pathogen. Subsequently, a more aggressive strain of Mt was isolated from hydrilla collected near Sheldon Reservoir in Texas and it has shown excellent potential as a biological control agent that could be applied alone or in combination with herbicides for hydrilla management (Shearer 1997, 1998; Netherland and Shearer 1996; Nelson et al. 1998; Shearer and Nelson 2002).

Constraints on the commercial development of microbial agents include the ability of the microorganism to form stable, infective propagules and the availability of a cost-effective method for producing propagules (Goettel and Roberts 1992, Wraight et al. 2001, Jackson 2007). Many plant pathogens including Mt are capable of producing sclerotia; i.e. melanized compact hyphal aggregates that survive desiccation and often serve as survival structures for the fungus (Cooke 1983, Coley-Smith and Cooke 1971, Shearer and Jackson 2006). Their stability and potential to produce infection in their host following germination make these propagules a promising form for use as a bioherbicide (Jackson and Schisler 1995, 2002). Microsclerotia of Mt have been shown to survive drying and can infect and kill hydrilla following rehydration (Shearer and Jackson 2006; unpublished results). Efficacy was not compromised after nine months in storage at 4° C (Shearer 2009b).

Temperature is an environmental factor that can impact growth of Mt in culture. Laboratory studies have shown that Mt will grow in broth culture at temperatures ranging from 20-34° C but not at 36° C (Jackson et al. 2011). Significantly higher amounts of biomass were produced at 24-30° C than at lower or higher temperatures. Colony forming units (cfu) peaked at growth temperatures between 20-24° C and microsclerotial production was highest when grown at 22-30° C. Effect of temperature on ability of air-dried microsclerotia to germinate hyphally and sporogenically was also assessed. After 5 months in storage, hyphal germination was consistently above 90% for cultures incubated between 24 and 30° C. Sporogenic germination (production of conidia) was greatest at incubation temperatures between 24 and 28° C.

In growth chamber studies, dried formulations of Mt have consistently reduced hydrilla biomass (Shearer 1998, 2009a, 2009b; Shearer and Nelson 2002); however, this has not always been the case with field studies. The major factors that would likely inhibit performance of Mt in the field are temperature, water flow, oxygen levels, and hydrilla canopy. Water temperatures fluctuate dramatically in the freshwater lakes, reservoirs, ponds, and canals of the southern United States (Bowes et al. 1979). During the summer, when hydrilla has developed a canopy, surface water temperatures may exceed 40° C, much beyond the optimal temperatures for Mt growth and survival. Flowing water would move the dried granules away from an application site, thus severely reducing the amount of inoculum required for hydrilla control in a particular area. Like most fungi, Mt is an aerobe requiring oxygen for growth. Low oxygen concentrations would adversely affect performance in the field. Finally, when hydrilla reaches the water surface it can form a dense canopy that would trap dried granules at the surface of the mat and not allow dispersion to sub-surface plant parts.

Field trials using dried Mt in Florida in the summer of 2007 were not conducted under moderate conditions but rather after hydrilla had formed a dense canopy and surface temperatures exceeded 30° C. (Jackson and Heilman 2008). In the June trial, water temperature data monitoring devices recorded surface temperatures greater than 30° C for the entire study period. This was also true for a subsequent August trial. Additionally, during this study the sub-canopy oxygen concentrations dropped to less than 1 mg L^{-1}. The near-anoxic water conditions observed in the test pond suggest that insufficient oxygen combined with high temperatures likely inhibited Mt growth.

To better understand selected factors that might compromise performance of dried formulations of Mt in the field, a pond study was designed to test effects of temperature, canopy, and hydrilla population densities on bioherbicide efficacy. To verify the viability and virulence of the fungal pathogen, Mt was first tested on hydrilla planted in aquariums in a growth chamber study. Mt was

then applied to the ponds at a rate that had been shown to significantly reduce hydrilla shoot biomass in the aquarium bioassay. The results of the aquarium and pond studies are reported herein.

MATERIALS AND METHODS

Fungal fermentation. Dry fungal inoculum for the study was provided by the United States Department of Agriculture, Agriculture Research Service, National Center for Agricultural Utilization Research, (NCAUR) Crop Bioprotection Research Unit, Peoria, IL. Microsclerotia were produced in a 100-L fermentation unit in a liquid culture medium developed by Shearer and Jackson (2006). The microsclerotia were separated from the fermentation broth using a rotary drum filter (Komline-Sanderson, Brampton, ON, Canada). The rotary drum filter was pre-coated with 4 kg diatomaceous earth (Hyflo Super Cell®, Celite Corporation, Lompoc, CA) to produce a 2.5-cm filter bed on the drum surface. Biomass of Mt was separated from the diatomaceous earth filter bed using a knife setting of 127 μm per drum revolution and a drum speed of 1 revolution min^{-1}. The wet filter cake obtained from the drum was ca 75% moisture. The wet filter cake was granulated using a conical mill (Quadro® Comil®, Quadro Engineering, Waterloo, ON, Canada) with a 6.4-mm screen and a rotor speed of 2,000 rpm. The wet granules of Mt were placed on aluminum baking trays at a depth of less than 2 mm and air-dried overnight to ca 30 % moisture. The moist granules were then passed through the conical mill two more times using a 2.4-mm screen followed by a 1.4-mm screen. Once air-dried to a moisture content of ca 6%, the fungal preparations were vacuum-packaged in polyethylene bags and stored at 4° C until used.

Aquarium study. An aquarium study was set up to test the viability and virulence of dried inoculum derived from two different Mt fermentation batches provided by NCAUR. The studies were conducted in 55-L aquariums located in a controlled-environment growth chamber at the U.S. Army Engineer Research and Development Center (ERDC). Conditions in the growth chamber were maintained for optimal hydrilla growth: 25 ± 1° C and a 14:10-hr light-dark photoperiod. The aquariums (0.9 m tall x 0.09 m^2) were filled with a water-based culture solution (Smart and Barko 1985). Osmocote (14-14-14) (Scotts-Sierra Horticultural Products Company, Marysville, OH) was placed in the bottom of 964-ml plastic cups (2.4 g per cup). Top soil (Earthgro, Hyponex Corporation, Marysville, OH) was added over the Osmocote to a depth of ca 21 cm and overlain with silica sand to prevent sediment and nutrient dispersion into the water. The cups were drenched with reverse osmosis water to wet the top soil. Five 15-cm apical cuttings from dioecious hydrilla were planted in each cup and placed in the aquariums (four cups per aquarium). Air was gently bubbled in to provide circulation. The plants were allowed to grow in the aquariums for approximately 28 days, by which time they had formed a canopy.

Dry inoculum was applied by scattering it evenly onto the water surface and allowing it to naturally dissipate over the hydrilla. As the rehydrated granules fell through the water column they became lodged on leaves and in leaf axils. Treatments included effective rates of 0.02, 0.04 0.06, and 0.08 g L^{-1} of both Mt batches and untreated controls. Each treatment was replicated three times. At 28 days after treatment, hydrilla shoot biomass was harvested, dried for 4 days at 60° C to a constant weight, and dry weight was recorded.

Pond study. Fifteen concrete ponds 5.5 m wide x 1.8 m deep were filled to a depth of 42 cm with top soil. Ammonium sulfate was applied at a rate of 2.3 kg per pond and carefully worked into the

soil. Alum-treated water from a holding pond was added to a depth of approximately 0.6 m above the soil surface. The ponds were allowed to temper for two months before they were planted to hydrilla in August to October 2009.

Hydrilla for the study was collected from a sump pond at the Aquatic and Wetland Ecosystem Research and Development Center at the ERDC. A total of 1,296 green healthy apical shoots of hydrilla (15 cm in length) were carefully picked for planting in each concrete pond. The shoots were placed in containers and covered with water and moist paper toweling to prevent drying of tissues. Two different planting densities, high and low, were used in the study. Eight ponds were planted at high density and seven ponds were planted at low density. For the high-density ponds, four single sprigs of hydrilla equally spaced were planted in the center of a 0.09-m^2 frame. The process was repeated 324 times until hydrilla shoots evenly covered the sediment surface. For the low-density ponds, sprigs were bundled together in bundles of nine and secured with a rubber band. Four bundles were planted equally spaced in the center of a 0.28-m^2 frame. The process was repeated 36 times. Water was added to a depth of 0.91 m.

In spring 2010, the ponds were carefully monitored for hydrilla growth. The goal was to treat the ponds with Mt early in the growing season when water temperatures were between 25 and 30° C. Because the ponds had been planted over a two-month period in the fall of 2009, hydrilla growth in early spring was not equal in each of the high-and low-density ponds; therefore, it was not possible to treat in May as planned. Treatment with Mt had to be delayed until July 2010. The ponds were covered with 30 % shade cloths to reduce water temperatures in the ponds. HOBO temperature probes (Onset Computer Corporation, Bourne, MA) were placed in each pond to monitor temperatures over the course of the study. Water was added to the ponds to a depth of 1.52 m. At the time of treatment, hydrilla was just beneath the water surface and had not topped out. Six high-density and six low-density ponds were selected for treatments in the pond study. Treatments were randomly assigned and included Mt applied to each of the two hydrilla densities at a rate of 3g/ft^2 of water surface area and untreated controls. Mt was hand applied and allowed to naturally dissipate over the hydrilla.

At 33 days post-inoculation, the ponds were drained in order to collect biomass samples. Ten randomly assigned replicate samples were collected from each pond using a 0.1-m^2 quadrat. Only aboveground biomass was collected. Each sample was washed to remove any soil and algae that had become attached to the plants. Excess moisture was removed by placing the samples in mesh bags and spinning on the spin cycle for six minutes in a washing machine with the agitator removed. The samples were then placed in paper bags, dried at 60° C to a constant weight, and dry weight was recorded.

Statistics. Analysis of variance (Anova) (Statistica Version 9.0 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 $P = 0.05$. One high-density control pond was not used in the statistical analysis because the mean biomass in grams was less than the mean biomass in two of the low-density control ponds.

RESULTS AND DISCUSSION:

Aquarium study: Two dried batches of Mt inoculum were applied at four different rates to 55-L aquariums planted to hydrilla. There were significant differences in the efficacy of the batches

($F_{1,19}$=27.213; p = 0.0001) and dose responses ($F_{3,19}$=10.892; p = 0.0002), but there was not a significant difference in the batch/dose interaction ($F_{1,16}$=2.4007; p = 0.10583) when compared statistically. The only Mt treatment that was significantly different from the others was batch 1 applied at 1 g (Figure 1). Because batch 2 produced in April performed better than batch 1 in the aquarium bioassays, it was chosen as the inoculum for the pond study. All dose rates of batch 2 significantly reduced hydrilla biomass in the aquariums when compared to untreated controls (F4,10=77.476; p = 0.0000) (Figure 2). Three of the four batch 2 treatments reduced shoot biomass by 100%. One batch being in storage for a month was probably not a factor because previous studies had shown that time in storage often increases efficacy of the pathogen (Shearer 2009b).

Pond study: At the time of inoculation, the high-density ponds had an even stand of hydrilla with percent cover between 95 and 100%. The low-density ponds had bare spaces between the hydrilla colonies with percent cover ranging between 70 and 75%. Hydrilla was ca 12 cm below the water surface of each pond at the time of application; thus, a dense topped-out plant canopy was not a factor in the experiment. Once the granules were wetted, they gradually dissipated through the water column. Mt is a contact pathogen and for infection to occur, the granules had to come in direct contact with the plants.

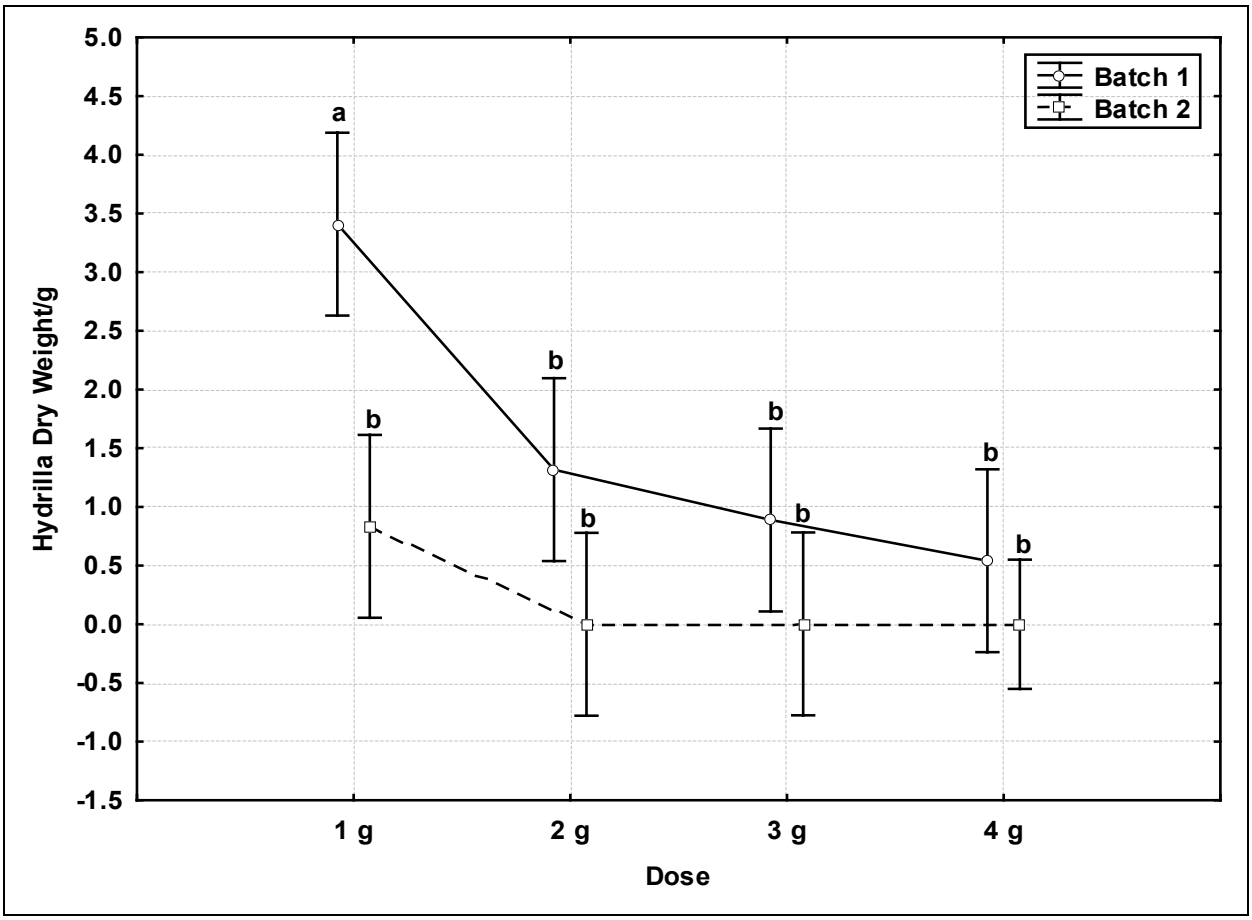

Figure 1. Shoot biomass of hydrilla treated with four different rates of Mt from two batches of inoculum. Batch 1 was produced in March 2010 and batch 2 was produced in April 2010. Bars having different letters are significantly different at p < 0.05.

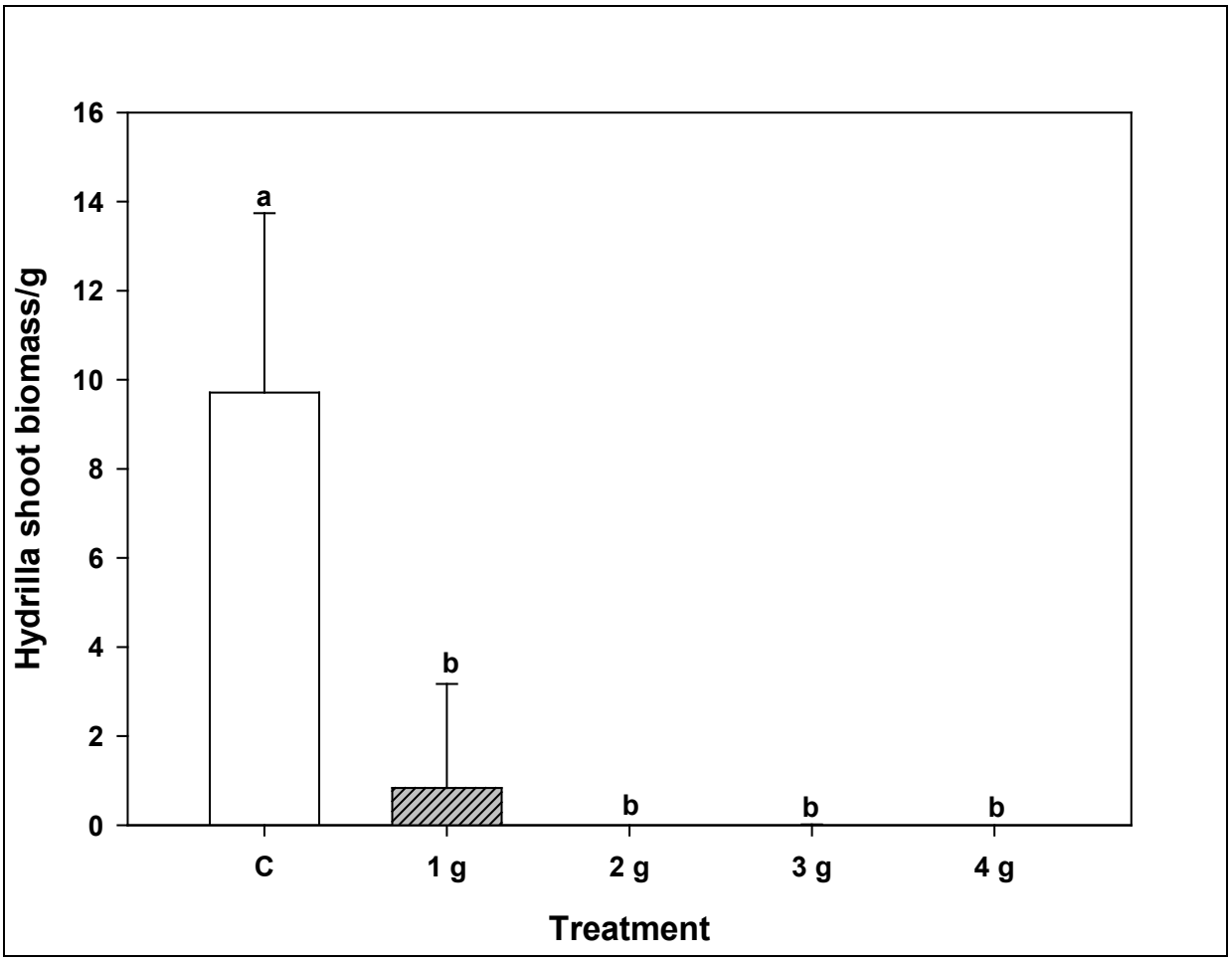

Figure 2. Shoot biomass of hydrilla treated with four different rates of dried Mt from a batch produced in April 2010. Bars having different letters are significantly different at $p < 0.05$. C = Control.

There was a significant interaction between planting density and treatment in the pond study ($F_{1,106}$=12.886; p =0.0005 (Figure 3). There was a 1.8-fold decrease in hydrilla biomass in high-density planted ponds treated with Mt compared to a 1.2-fold decrease in hydrilla biomass in low-density planted ponds. There was no significant decrease in biomass between the control and the Mt-treated low-density planting, but there was a significant decrease between the control and the Mt-treated high-density planting. Although each pond was planted using the same number of plants, at the time of harvest, the high-density control ponds had a 2.6-fold higher biomass than the low-density control ponds. In the high-density planted ponds, the plants were evenly spaced so there were greater chances of more granules impinging on plant surfaces than with the low-density planting, where the individual plants were more tightly clumped together and the spacing did not allow the granules to equally impinge on plant surfaces. In terrestrial plant studies, Burdon and Chilvers (1982) found that an increase in host density increases the number of target plants and with it the probability that a unit of inoculum will be intercepted. Additionally reducing the distance between adjacent plants reduces the chance of inoculum being lost to the ground. Two other factors may have contributed to the difference in planting density and treatment success in the pond study. As mentioned before, the low-density planted ponds had clumps of hydrilla with spacing between the clumps. Over the course of the experiment, hydrilla could have rapidly expanded into the bare spaces and these newly established

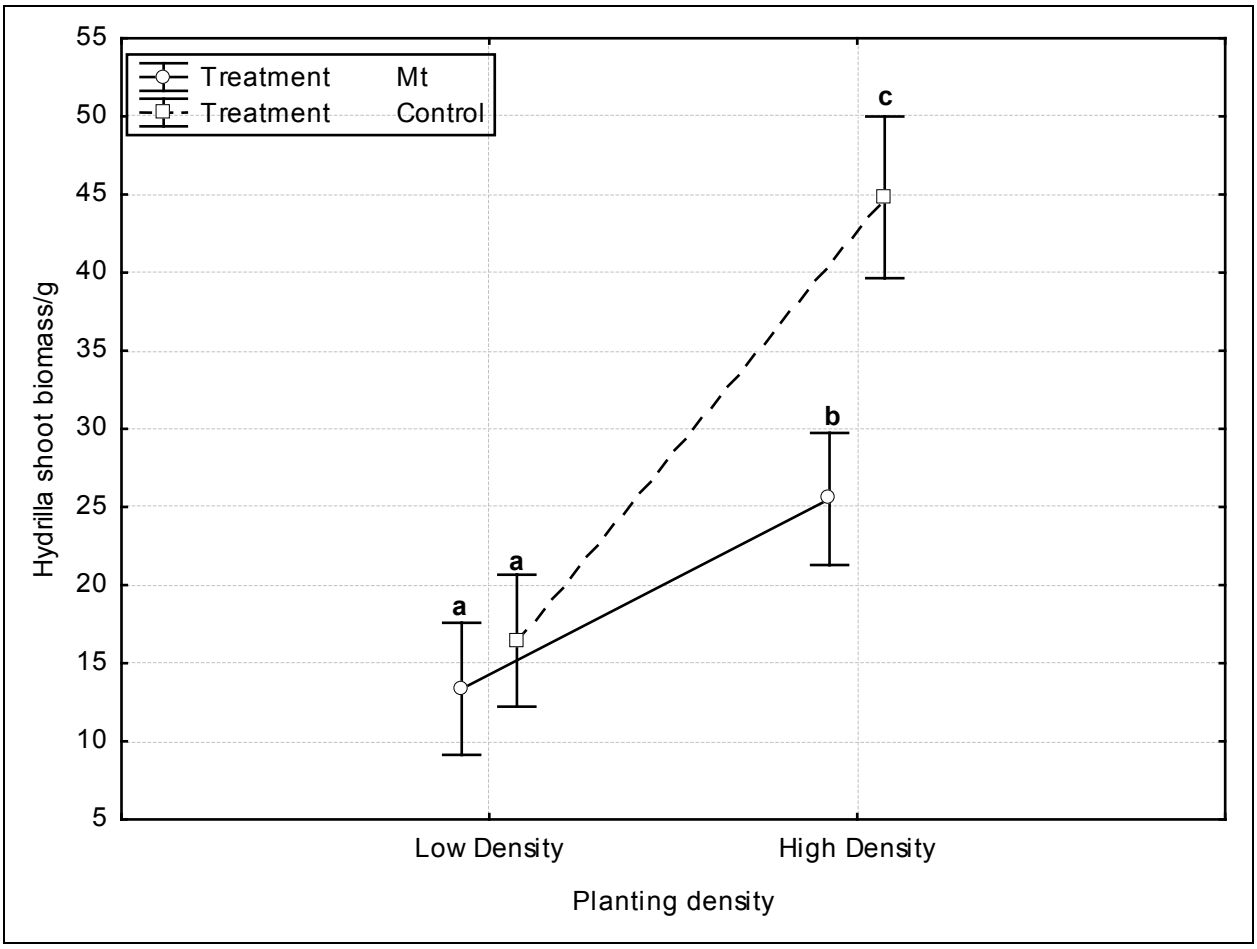

Figure 3. Shoot biomass of hydrilla harvested 33 days post-inoculation with dry granules of *Mycoleptodiscus terrestris* applied at a rate of 3g/ft^2 surface area to ponds planted at two different densities. Bars having different letters are significantly different at $p < 0.05$.

plants would not have received any inoculums, thus affecting the final results. Secondly, the results could possibly have been inadvertently affected by the biomass harvest. The ponds were drained prior to harvest and in so doing, the plants would no longer have been vertical but would have fallen over and become strewn horizontally over the sediment surface, potentially covering any bare spots that remained. Inoculum application also did not contribute to the differences because in all treatments there was equal coverage of granules over the surface of each pond.

The experiment results indicate that certain factors (e.g. plant density and temperature) can reduce pathogen efficacy. With prior knowledge of the effects these various factors can have on Mt performance, field applications could be timed to minimize their impact. For example, applying Mt early in the growing season would help negate the effects of temperature. However, due to uneven plant growth in the low-and high-density planted ponds, the study did not commence until mid-July, two months later than originally planned. The record high temperatures recorded in Vicksburg, MS during July and early August caused pond temperatures to rise to the point where they could inhibit growth and survival of Mt. The Mt strain that is presently being used performs best at temperatures below 30 °C and although the mean temperature in the ponds was ca 29 °C, the temperature recorders indicated that water temperatures did exceed 30 °C during the warmer parts of the day.

Adjusting the formulation or the fermentation parameters could possibly increase performance in the field. For example, the nitrogen source used in the fermentation broth affects the number of microsclerotia produced in the medium (Shearer and Jackson 2006). Adjuvants incorporated into the formulation or alternatively added at the time of application might allow better adhesion of the granules to plant surfaces. Granule size could also be reduced, allowing greater numbers of particles per gram of the bioherbicide to potentially impinge on leaf surfaces. One other distinct possibility for improvement of the pathogen would be to identify or select for a strain that tolerates higher water temperatures.

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