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"Laboratory and Field Demonstration of weed control properties of dry formulations of *Mycoleptodiscus terrestris,* a potential fungal bioherbicide for control of *Hydrilla verticillata*"

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ABSTRACT
Studies focused on determining optimized nutritional and environmental conditions for scale-up of the production of microsclerotia of *Mycoleptodiscus terrestris* (MT) using deep-tank fermentation and assessing the temperature compatibility of MT. We evaluated the impact of temperature, pH, aeration, agitation, and nitrogen source on MT growth and microsclerotia production. Fermentation studies comparing nitrogen sources showed that microsclerotia yields were higher in MT cultures grown with hydrolyzed cottonseed meal when compared to cultures grown with corn steep liquor powder. Aeration and agitation studies showed that higher aeration rates and agitation speeds that increased dissolved O_2 levels reduced MT fermentation times and increase microsclerotia yields. Growth temperatures between 26-28 ºC were optimal for biomass accumulation whereas temperatures of 20-24 ºC optimized colony forming unit development in liquid cultures of MT. Microsclerotia formation likely reduces cfu counts leading to the discrepancy in optimal temperatures for biomass and cfu development. Studies with air-dried MT microsclerotia showed that temperatures of 24-28 ºC were required for both hyphal germination and conidia production. Neither MT biomass nor air-dried microsclerotia germinated or grew at temperatures above 32 ºC increasing the safety of the biocontrol agent for humans and other mammals while reducing the potential of MT for use as a biocontrol agent. Bioassays against hydrilla with MT microsclerotia were conducted in laboratory, mesocosm, and small-scale field trials. Bioassay results were mixed using various formulations of MT microsclerotia as a stand alone product and in integrated studies with fluridone. In the 4 small-scale field tests conducted in Osceola County from late May until mid November, only the May trial showed significant levels of hydrilla biocontrol activity. While reductions in hydrilla biomass accumulation were noted in the other trials, statistical differences were not observed. These results suggest water temperature may play a role in the biocontrol efficacy of MT against hydrilla.

INTRODUCTION

The fungal pathogen *Mycoleptodiscus terrestris* (MT) remains to be fully developed as an operational tool for hydrilla management. The commercial scale-up of fermentation methods already developed for MT microsclerotia (Shearer and Jackson, 2006) that enhance MT virulence on hydrilla are needed. In addition, the impact of temperature on MT growth and sporulation requires evaluation to determine the optimal fermentation temperature and appropriate water conditions for the application of MT microsclerotia as a hydrilla biocontrol agent. The ultimate goal of these efforts is the commercial adoption of the MT technology for hydrilla control.

During the first year of this study, we focused our efforts on the optimization of pilot-scale nutritional and environmental conditions for MT biomass and microsclerotia production, conducted bioassays and field trials against hydrilla using fermentor-produced MT, and evaluated temperature constraints to the application of MT microsclerotia preparations. A commercial fermentation consultant was enlisted to evaluate our process and suggest fermentation process parameter critical for scale-up to the commercial fermentation level. We have been in discussions with contract fermentation facilities concerning the large-scale production of MT but have not contracted their services at present. Reported are results from these studies.

MATERIALS AND METHODS

Strains and culture conditions

Mycoleptodiscus terrestris (Gerd) Ostazeski Starin TX-05 (NRRL 30559) was used in these field studies and was isolated from infected hydrilla (*Hydrilla verticillata*) in Sheldon Reservoir, Texas. Stock cultures of *M. terrestris* (MT) were grown on potato dextrose agar (PDA) plates for three weeks at room temperatures, cut into 1 mm^2 agar plugs and stored in 10% glycerol at -80 ºC. Inocula for biomass production were obtained from PDA plates inoculated with a frozen stock culture of MT and grown at room temperature (~25 ºC) for 2 weeks, as described previously (Shearer & Jackson, 2006).

For microsclerotia production, 100 or 400 mL cultures of MT were grown on pre-culture media in 250mL or 1L, baffled, Erlenmeyer flasks at 28C and 300 RPM in a rotary shaker to obtain hyphal inoculum. Shake-flask and 5L fermentor microsclerotia production cultures were inoculated with a 10% inoculum of the MT pre-cultures. Three liter pre-cultures of MT were used for inoculation of the 100L fermentors.

For small-scale fermentations, 5L fermentors (B Braun BiostatB) were used with 4L working volume. Optimal aeration and agitation rates were determined to be 2L min^{-1} and 600 RPM. Fermentor conditions for MT microsclerotia production in the 100L fermentor were 28 ºC, aeration rates of 50 liters sterile air $minute^{-1}$, and an agitation rate of 350 RPM. Five liter and 100L fermentors were harvested 4 days post inoculation. For quality assurance, harvested fermentation broth was streak plated on nutrient agar and incubated at 30 ºC for 2 days to ensure that the fermentation was not contaminated. Only uncontaminated MT cultures were harvested from the fermentation process and used in formulation studies or bioassays and field trials for hydrilla control.

Media Composition and Fermentation Conditions

The basal component, trace metals and vitamins of the media used for MT pre-culturing and MT microsclerotia production have been previously described (Shearer & Jackson, 2006). Standard microsclerotia production media was adjusted to an initial pH of 5.5 and pH was uncontrolled during culture growth. In pH studies, media was adjusted to pH 5.5 and 4.5 and was controlled with 2N HCl and 2N NaOH. Glucose was provided as a carbon source at concentrations of 20 g/L for pre-culture media and 60 g/L in microsclerotia production media. The nitrogen source used for the MT pre-culture media was 30 g/L hydrolyzed cottonseed meal and for the microsclerotia production media was 30 g/L corn steep liquor powder. In 5L fermentor studies, various concentrations of hydrolyzed cottonseed meal were tested as a nitrogen source for microsclerotia production and compared to standard microsclerotia production medium. The impact of pH on microsclerotia formation and yield was also examined.

Harvest and Drying Protocols for *M. terrestris* Microsclerotia

The microsclerotia-containing MT biomass was separated from the fermentation broth using a rotary drum vacuum filter. The rotary drum vacuum filter was pre-coated with 4 Kg diatomaceous earth 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 microns per drum revolution and a drum speed of 1 revolution $minute^{-1}$. The MT microsclerotia filter cake was ~ 75% moisture. The filter cake was granulated, placed in shallow trays at a depth of less than 2 mm, and air-dried overnight to ~ 30% moisture. The particle size of the filter cake was further reduced in size to ~ 250 microns. Once air-dried to less than 8% moisture, the microsclerotia-containing diatomaceous earth preparations were vacuum-packaged in polyethylene bags and stored at 4 ºC.

Dried MT preparations were tested for purity and spore production capability by sprinkling 25 mg onto water agar plates. Plates were incubated for 24 hours to assess viability (hyphal germination) and 8 days for aerial conidia production.

Temperature Tolerance Studies

Temperature compatibility studies were conducted with hyphal biomass and air-dried microsclerotia preparations of MT. For hyphal biomass studies, MT biomass was produced in pre-culture media using the previously described liquid culture methods. Microsclerotia production media was inoculated with 10% hyphal biomass from pre-cultures and incubated at various temperatures (20 to 36 ºC in 2 ºC increments) for 8 days at 300 RPM in a rotary shaker incubator. Biomass accumulation was measured on days 0,1,2,3 by dry weight determination, as previously described (Shearer & Jackson, 2006). Colony forming units (cfu) were determined for each treatment by streaking 1 mL of an appropriate dilution of each replicate sample onto Martin's agar plates, a medium selective for MT (personal communication, JF Shearer; Baruch & Stack, 1990). The plates were incubated for 24-48 hours at 28 ºC and a stereoscope was used to determine the total number of colonies forming on each plate. All treatments were replicated 8 times in each experiment and all experiments were performed in time at least twice.

For temperature studies with air-dried microsclerotia preparations, 25 mg of the air-dried microsclerotia was sprinkled onto water agar plates that had been pre-incubated at the incubation temperature to be tested. The temperatures tested were 20, 22, 24, 26, 28, 30, 32, 34, and 36 ºC. Hyphal germination and conidia production were measured. All treatments were replicated 8 times and all experiments were performed at least twice.

Hydrilla Bioassays

Air-dried and wet, whole cultures of MT were given to Drs. Chris Dunlap, USDA for formulation studies or shipped on ice by overnight freight to Dr. Mark Heilman, SePRO Corporation, Whitackers, NC or Osceola County, FL and/or to Dr. Judy Shearer, USACE, Vicksburg, MS for laboratory bioassays or field trials against hydrilla. USDA provided test materials to SePRO and US Army ERDC to perform three mesocosm trials since the final approval of the QAPP in mid May.

RESULTS & DISCUSSION

Aeration studies using 5L and 100L fermentors showed that higher aeration rates, due to increased aeration or increased agitation, enhanced the rate of biomass accumulation, microsclerotia production, and microsclerotia melanization (data not shown). Agitation rates of 600 RPM and aeration rates of 2 L air min^{-1} were optimal for fermentations in the 5L fermentors and agitation and aeration rates of 350 RPM and 50 L air min^{-1}, respectively, were optimal for microsclerotia formation and melanization in the 100L fermentors. A reduction in fermentation time translates into reduced production costs for microsclerotia of MT. The impact of the sheer associated with agitation rate continues to be evaluated as this may have an impact on microsclerotia formation, stability and size. Studies using 5 liter fermentors showed that microsclerotia production was increased when Pharmamedia® was used as the nitrogen source (Table 1). Interestingly, increased microsclerotia production by Pharmamedia®-containing media did not result in better hydrilla control in lab bioassays (Data not shown).

Table 1. Comparison of biomass and microsclerotia production by *M. terrestris* cultures grown in a basal salts medium with 6% glucose and dried corn steep liquor (Solulys®) or cottonseed meal hydrolysates (Pharmamedia®). Four liter cultures grown in a 5L fermentor (Biostat B) at 600 rpm and 2 standard liters air $minute^{-1}$.

Nitrogen Source – [g/L]	mg Biomass mL^{-1} (+/- Std Err)	Microsclerotia $mL^{-1} \times 10^3$ (+/- Std Err)
Solulys® - 3%	37.5 (5.1)	6.2 (2.1)
Pharmamedia® - 3%	34.5 (0.7)	13 (8.0)
Pharmamedia® - 2%	31.8 (1.2)	11 (3.3)
Pharmamedia®	35.3 (1.4)	12 (2.6)

Evaluation of the influence of pH on biomass accumulation and microsclerotia production was conducted with 4L fermentations in the 5L fermentors. Studies focused on controlling pH values at 4.5 and 5.5 during the growth of MT and comparing results to standard microsclerotia production in which pH is adjusted to 5.5 and uncontrolled during fermentation. These studies showed that biomass accumulation and microsclerotia formation was unaffected by pH (data not shown). Future studies will compare the bioefficacy of microsclerotia produced in MT cultures grown at pH 4.5 against hydrilla as fermentation at low pH significantly reduces the potential for bacterial contamination, an important consideration in large-scale fermentation.

A meeting was held with a former Eli Lilly fermentation expert to consult in evaluating current production processes for MT microsclerotia. This meeting was initiated due to differences in the bioactivity of MT biomass produced in shake-flasks when compared to fermentors. The outcome of the meeting suggested that there are some critical aspects of the production process (aeration, agitation, inoculum development) that must be determined before scale-up of the fermentation process continues. Studies have been initiated to evaluate these parameters.

Temperature compatibility studies were conducted using hyphal biomass inoculum of MT and with air-dried, microsclerotia preparations. Hyphal biomass studies were evaluated for growth in nutritive media at temperatures from 20-36 ºC. Biomass accumulation (dry weight, mg/mL) and cfu were used as a measure of growth. Steady increases in cfu were seen when cultures were incubated at 20-26 ºC (Figures 1). Cultures incubated at 28 ºC did not see an increase in cfu after 3 days growth, presumably due to the formation of microsclerotia. The cfu level for MT cultures grown at 34 and 36 ºC declined daily with a complete loss of viability for cultures grown at 36 ºC by day 7 (Figure 1). Temperatures above 32 ºC inhibit the growth and survival of MT. The formation of microsclerotia, in which numerous fungal hyphae aggregate, confounds the use of cfu as a measure of growth for MT cultures. The steady increase in cfu by cultures grown at 20-26 ºC suggests that microsclerotia are not formed by these cultures or that smaller sclerotial bodies are formed. Evaluation of biomass accumulation at the various growth temperatures showed that cultures grown at 26-28 ºC accumulated the highest amount of dry weight (Figure 2). When combined, these studies suggest that the growth of MT is maximized at 26-28 ºC. Further studies are needed to determine the level of sclerotia formation at 26 ºC when compared to standard production at 28 ºC.

The evaluation of germination and conidia production by air-dried microsclerotia following rehydration and incubation at various temperatures is informative from a safety and product use standpoint. Air-dried microsclerotia did not germinate when rehydrated and incubated at 36 ºC (Figure 3). These results coupled with the liquid cultures studies suggest that MT cannot grow at human body temperature. Results also suggest that the use of MT at temperatures of 32 ºC or greater reduced microsclerotia germination. Conidiation of microsclerotia of MT is significantly impacted by incubation temperature (Figure 4). No conidiation occurred when microsclerotia were incubated at temperatures below 22 ºC or above 30 ºC. The production of conidia by MT is believed to increase hydrilla infection suggesting that maximum biocontrol efficacy would occur when water temperatures are 22-30 ºC.

Bioassays and small-scale field test of formulated and unformulated MT preparations were conducted May 25 (SePRO), June 21 (ERDC), and August 2 (SePRO) in conjunction with small-scale field studies in Osceola County, FL. Results of the May 25th bioassay in 60L aquaria are shown in figure 5. Formulated and unformulated preparations of MT were applied at 25 and 50 mg per liter of water. At the low test rate, some MT dry materials were rehydrated for 6 hours prior to application. Statistical evaluation indicated a near-significant treatment effect ($p=0.058$) but ultimately no significant differences between block populations ($p=0.392$). In terms of hydrilla control, rehydrated materials had less activity than MT materials applied dry and there were no clear rate differences. The May 25th small-scale field trial in Osceola County was not successful due to either an enclosure method modification or heavy early June rain associated with Tropical Storm Barry. Hydrilla biomass collection at one month post treatment showed no reductions from treatment with MT (data not shown).

In late June, ERDC cooperator, Dr. Judy Shearer, conducted aquarium mesocosm study in conjunction with the late June field trial. The mesocosm and field study included interaction with fluridone (trade name Sonar) as an integrated component. Fluridone has been documented to interact synergistically with MT in controlling hydrilla. The results from the mesocosm study indicated better than 80% hydrilla biomass reduction at an application rate of 20 mg/L dried MT and improved injury in combination with 10 ppb fluridone (Figure 6). In the June 21, 2007 Osceola County field trial, one-month and 2.5-month post harvests demonstrated this synergism as enclosures treated with *M. terrestris* and Sonar showed improved reductions in hydrilla biomass versus enclosures treated with either product alone (Figure 7). This interaction had never been previously documented under field conditions. Results from these field studies showed less hydrilla control with MT compared to an early spring pond bioassay (data not shown). The reduction in biocontrol activity against hydrilla was likely due to elevated water temperatures and other water quality stress associated with vertical stratification in mature hydrilla beds. As shown in our temperature tolerance studies, MT does not grow well above 30 ºC, and concurrent water temperature data monitoring determined surface water temperatures exceeded this level for most of study period. Visual observations also suggested that hydrilla injury was greater in shoot sections below hydrilla mat where ambient water temperatures were cooler.

On August 1, 2007, mesocosm studies were initiated at SePRO's Whitackers, NC facility to support the Osceola County field trial initiated August 2. Results of these mesocosm studies showed less injury to hydrilla when compared to Dr. Shearer's late June study (data not shown), likely due to static conditions or the higher water temperature of outdoor test tanks versus the actively aerated ERDC aquaria. At a 5 ppb fluridone dose, results did not indicate interaction between MT and fluridone in this trial. In an effort to improve MT performance for the Osceola County field trial, shearers were used to remove the top 18 inches of hydrilla mat in test enclosures one week prior to treatment. The goal here was to improve water exchange within treatment plots and allow MT product to settle into cooler zone where it would likely be more active. Biomass harvested one-month post inoculation showed no significant treatment effects (data not shown), and water temperature data suggested that pre-treatment canopy removal did not enhance mixing within enclosures. In addition, dissolved oxygen monitoring suggests that since early August, sub-canopy oxygen concentrations in the trial pond dropped to less than 1 mg per liter. Like most fungi, MT is an aerobe requiring oxygen for growth. The near-anoxic water conditions observed in the test pond suggest that insufficient oxygen and high temperatures likely inhibited MT growth.

In conjunction with the November16 Osceola County field trials, an aquarium-scale mesocosm trial was initiated at SePRO, Whitakers, NC. Tanks with hydrilla were treated on November 20 with formulated and unformulated MT bioherbicide prototypes. Results showed significant treatment effect but failed to indicate significant differences between treatments despite a trend towards greater biomass reduction at higher test rates (data not shown). Results from the November 16th field trial in Osceola County showed a trend of biomass suppression at one-month post-treatment but a lack of statistically significant reductions and clear recovery at two months post-treatment (Figure 8).

ERDC cooperator Judy Shearer conducted a comparison of various stored, dry MT preparations from spring and summer production runs that were used in bioassays and field trials. Her aquarium mesocosm tests indicate improved performance by dried MT preparations following 3-7 months storage at 4 ºC (Figure 10). In general, all MT materials used in mesocosm and Osceola County trials were produced and used fresh with little lag time between production, drying, and use. The improved biocontrol efficacy of these preparations suggests that a storage or maturation period for dried MT microsclerotia preparations may improve the bioefficacy of this bioherbicide.

CONCLUSIONS

These studies have demonstrated that hydrilla can be infected and killed with MT under field conditions but also demonstrated that this control was inconsistent under the conditions of this study. Consistent hydrilla control with MT will be possible only when we have a better understanding of the interaction between MT, the environment, and hydrilla. Our temperature studies with MT showed the critical importance of water temperature to MT growth, sporulation, and, presumably infection of hydrilla. Many of our field studies were conducted when water temperatures out of range for the growth and sporulation of MT. A better understanding of the optimal timing for MT application to hydrilla should lead to more consistent hydrilla control with MT. The commercial development of MT as a bioherbicide will require, not only consistent hydrilla control in the field, but also an efficient, economical method of MT production. We have made inroads to understanding the process constraints that must be addressed to produce effective MT preparations for hydrilla control using deep-tank fermentation. Agitation, aeration, pH, nitrogen source can all impact the formation and yield of microsclerotia of MT. Future research will focus on 1) an understanding of the environmental constraints (agitation, aeration, pH) to the scale-up of the MT fermentation process, 2) identification of a contract fermentation firm to carry out large-scale MT production for expanded field trials, 3) evaluation of water temperature for MT application for optimal biocontrol efficacy using controlled condition bioassays, and 4) optimization of MT application timing for use in controlling hydrilla in the field.

REFERENCES

Shearer, JF, Jackson, MA. 2006. Liquid culturing of microsclerotia of *Mycoleptodiscus terrestris*, a potential biological control agent for the management of hydrilla. Biological Control. **38**: 298-306.

Baruch, S., Stack, J. 1990. Selective Medium for Isolation of *Mycoleptodiscus terrestris* from Soil Sediments of Aquatic Environments**.** Applied and Environmental Microbiology **56**: 3273-3277.

Figure 1

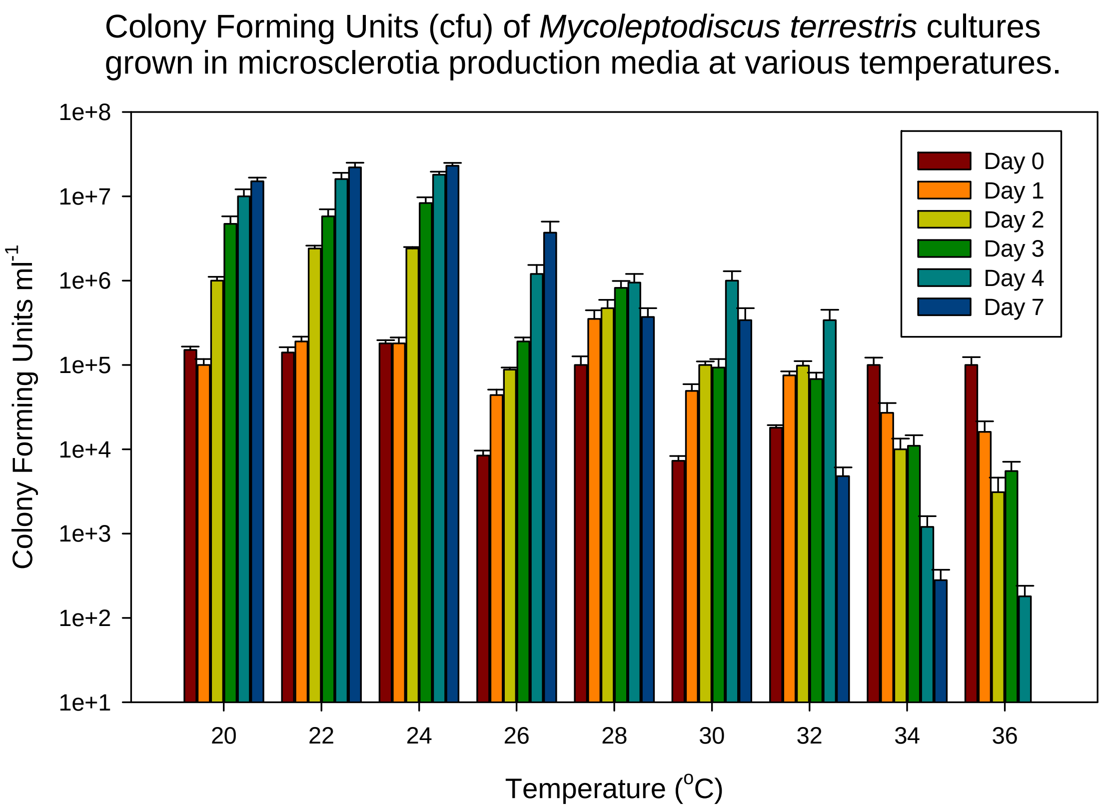
Colony Forming Units (cfu) of *Mycoleptodiscus terrestris* cultures grown in microsclerotia production media at various temperatures.
Colony Forming Units ml-1
Temperature (oC)
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
20
22
24
26
28
30
32
34
36
Day 0
Day 1
Day 2
Day 3
Day 4
Day 7

Figure 2

Biomass accumulation by cultures of *M. terrestris* grown in microsclerotia production media at various temperatures. Hyphal inoculum 10 mg biomass/ml (----), 3 days incubation at various temperatures and 300 RPM in a rotary shaker incubator

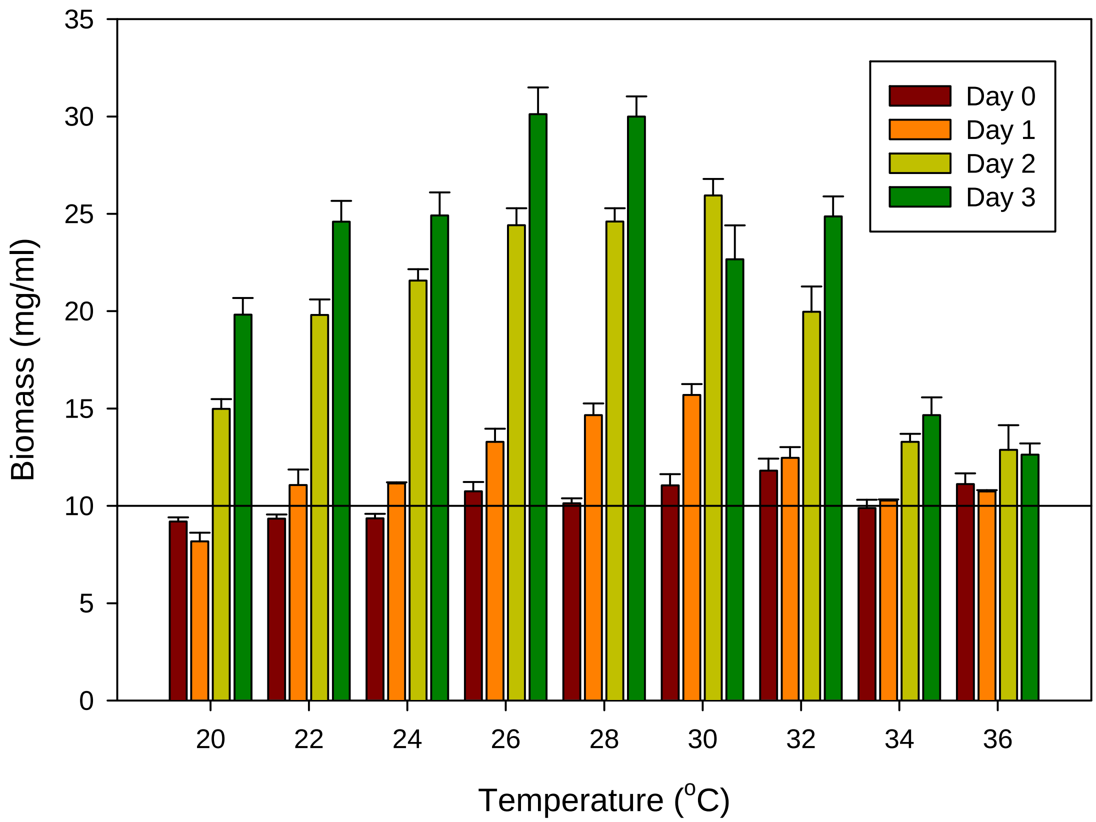

Figure 3

Germination of air-dried microsclerotia of *M. terrestris* after rehydration and incubation on water agar plates for 24 hours at various temperatures

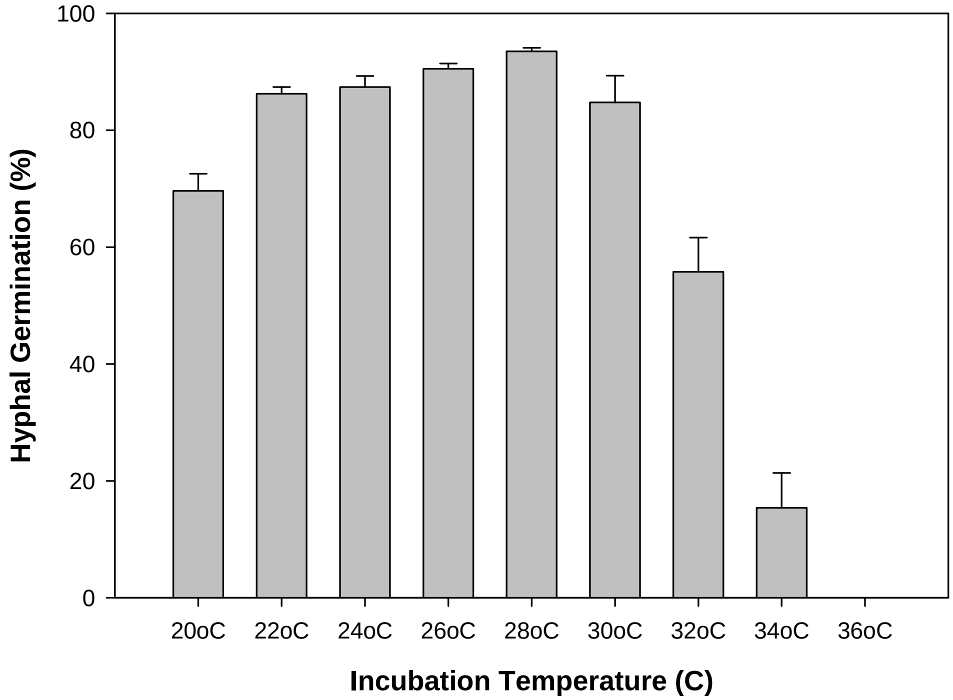

Figure 4

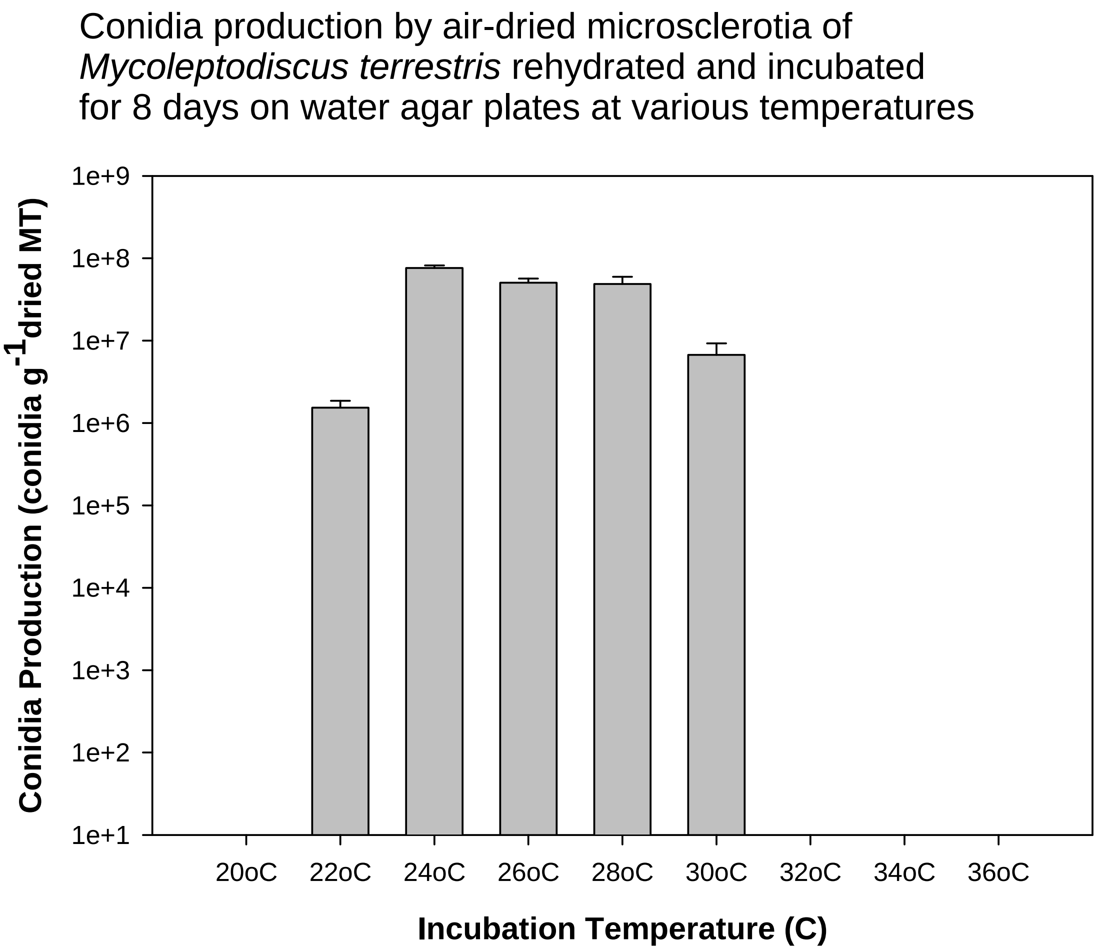
Conidia production by air-dried microsclerotia of
Mycoleptodiscus terrestris rehydrated and incubated
for 8 days on water agar plates at various temperatures
Conidia Production (conidia g-1dried MT)
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
20oC
22oC
24oC
26oC
28oC
30oC
32oC
34oC
36oC
Incubation Temperature (C)

Figure 5. Mesocosm companion study to May 25th Osceola County pond study at SePRO, Whitackers, NC

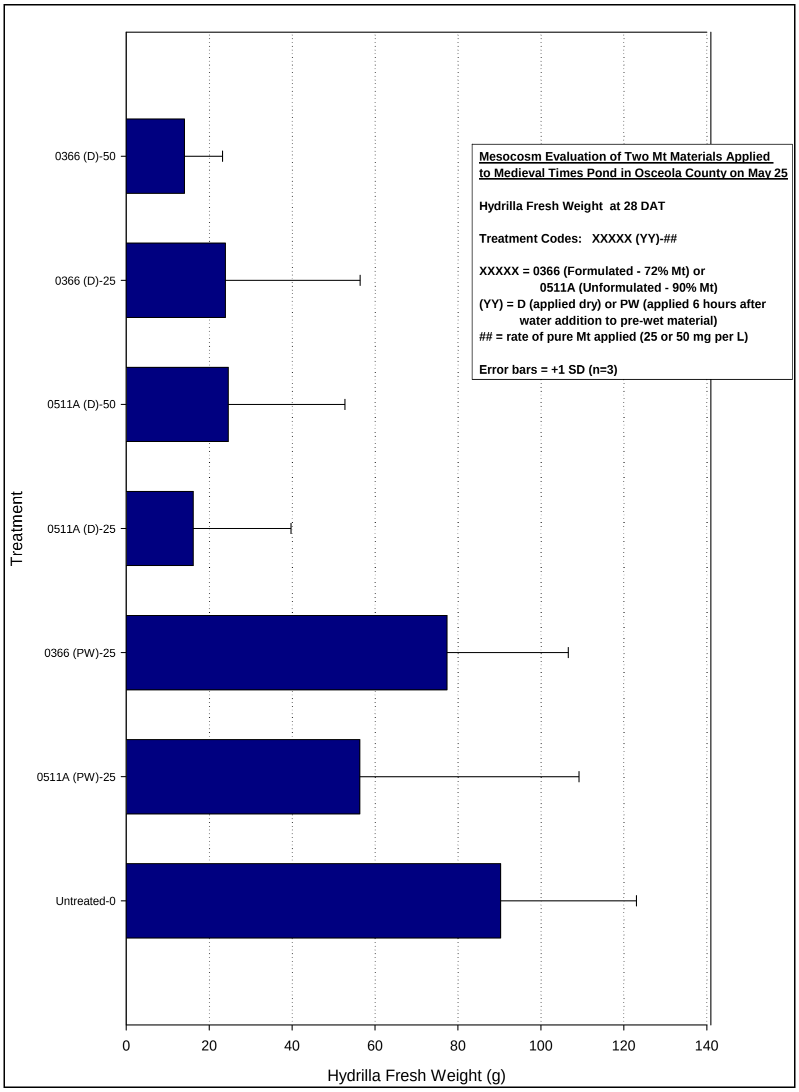

Figure 6 Companion mesocosm study (Dr. Judy Shearer) of *M. terrestris* preparations used in June 21 Osceola County pond studies

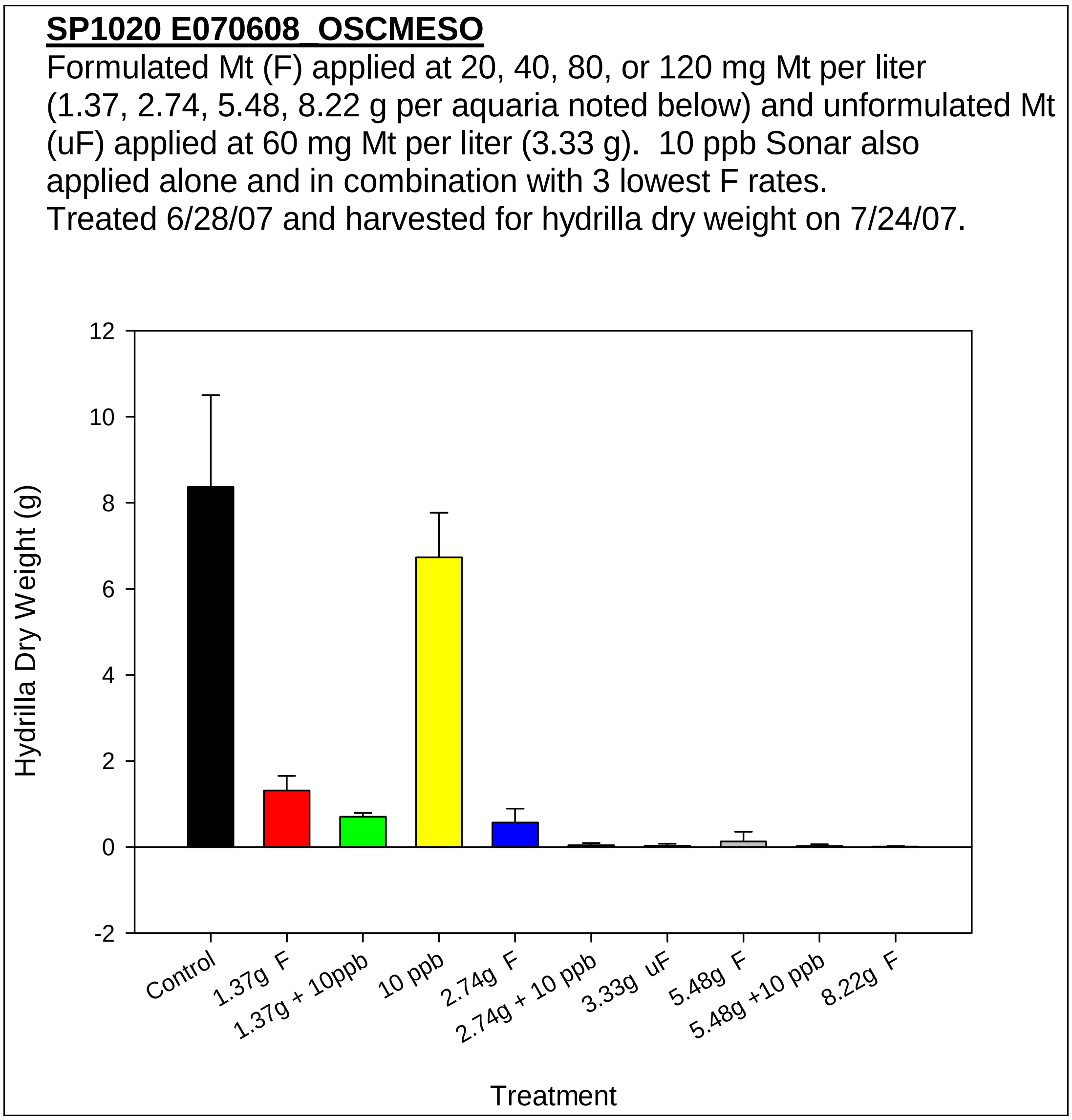

Figure 7. June 21 Osceola County pond field bioassay with *M. terrestris* preparations for control of hydrilla

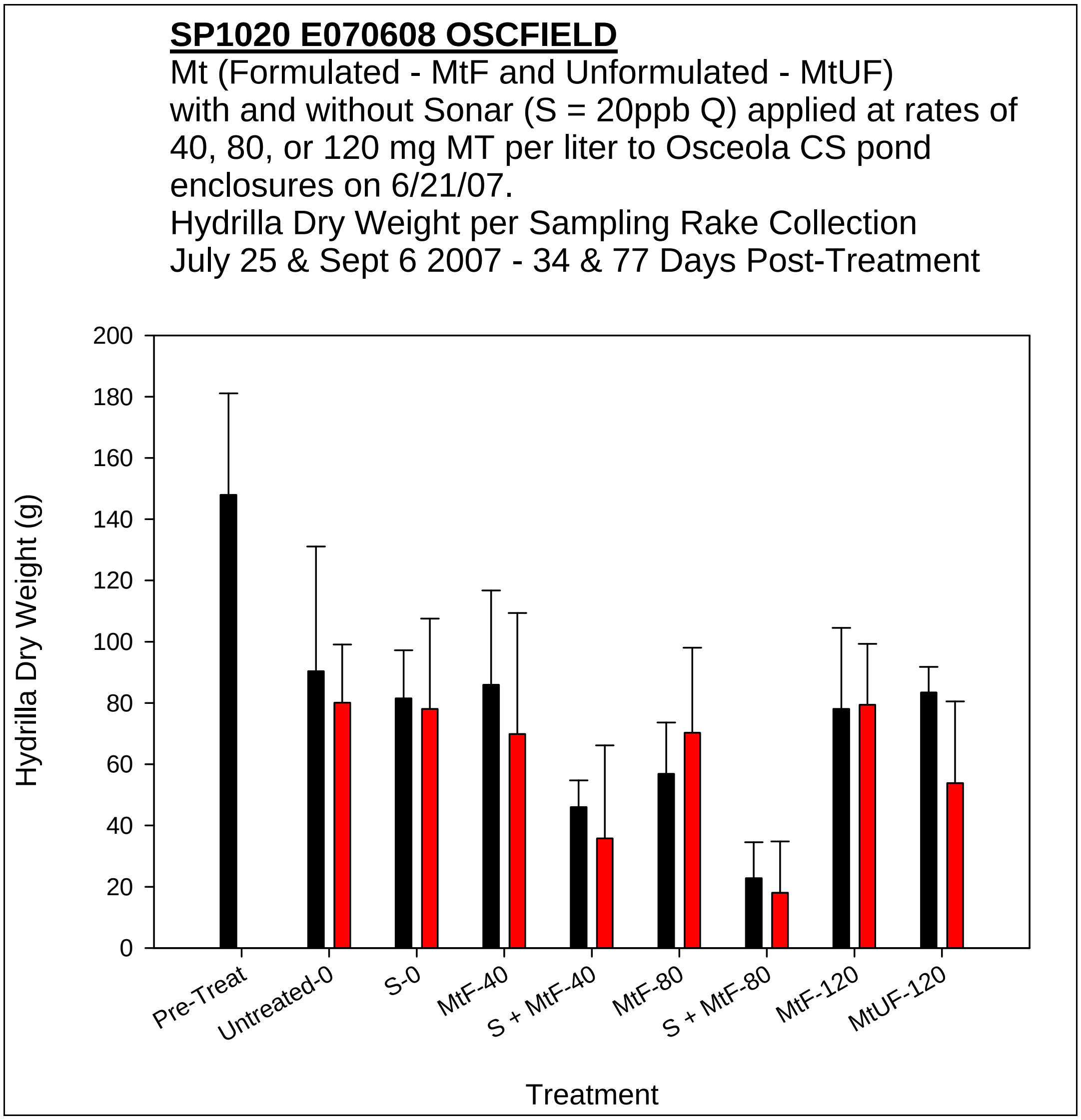

Figure 8 November 16 field trials in Osceola County. M. terrestris microsclerotia were applied to hydrilla containing pond enclosures.

SP1020 OSCFIELD 2007Nov16

Hydrilla shoot dry weight sampled from test enclosures at Osceola County field site 26 and 62 Days after treatment on Nov 16. Plots treated with formulated (F) and unformulated (UF) Mt (USDA E071017) at rates of 40, 80, 160, 240 mg active Mt (F) per liter and 160 mg active Mt (UF) per liter. ANOVA failed to detect statistically significant treatment effect at 26 DAT (p=0.076) or 62 DAT (p=0.848). Pre-Treatment not included due to difference in collection rake design.

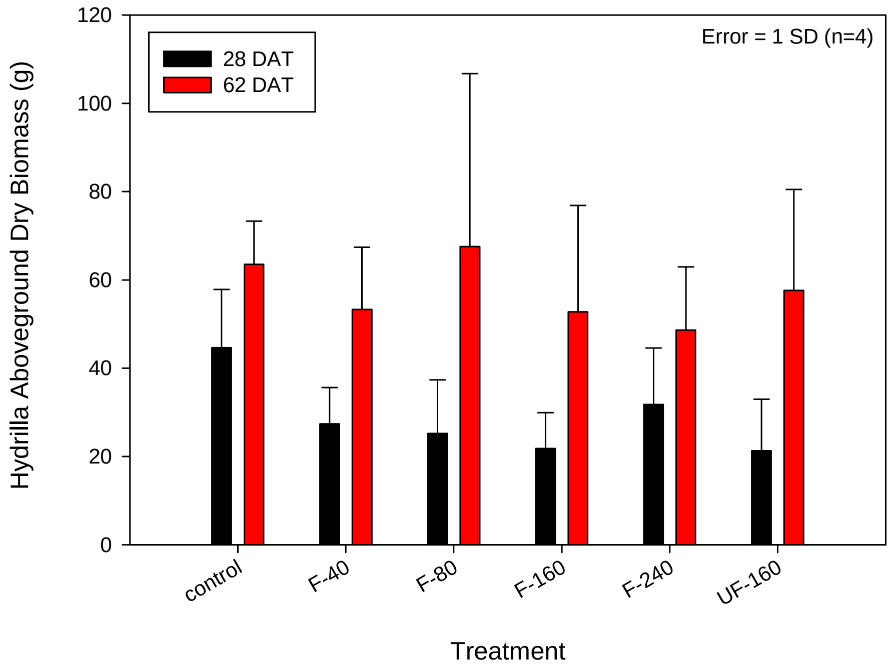

Figure 9. November 16 hydrilla bioassay mesocosm studies run at SePRO's Whitackers, NC facility using M. terrestris preparations used in the Osceola County field trial.

SP1020 OSCMESO 2007Nov20

Hydrilla shoot dry weight in SePRO RTC Static Outdoor Mesocosms at 28 DAT. Tanks treated 11/20/07 with formulated (F) and unformulated (UF) Mt (USDA E071017) at rates of 17, 34, 68, 136, 204 mg active Mt (F) per liter and 76 and 152 mg active Mt (UF) per liter. ANOVA indicates significant treatment effect ($p<0.001$). Holm-Sidak comparison test detected significant differences between untreated control and treatments but did not detect differences between treatments.

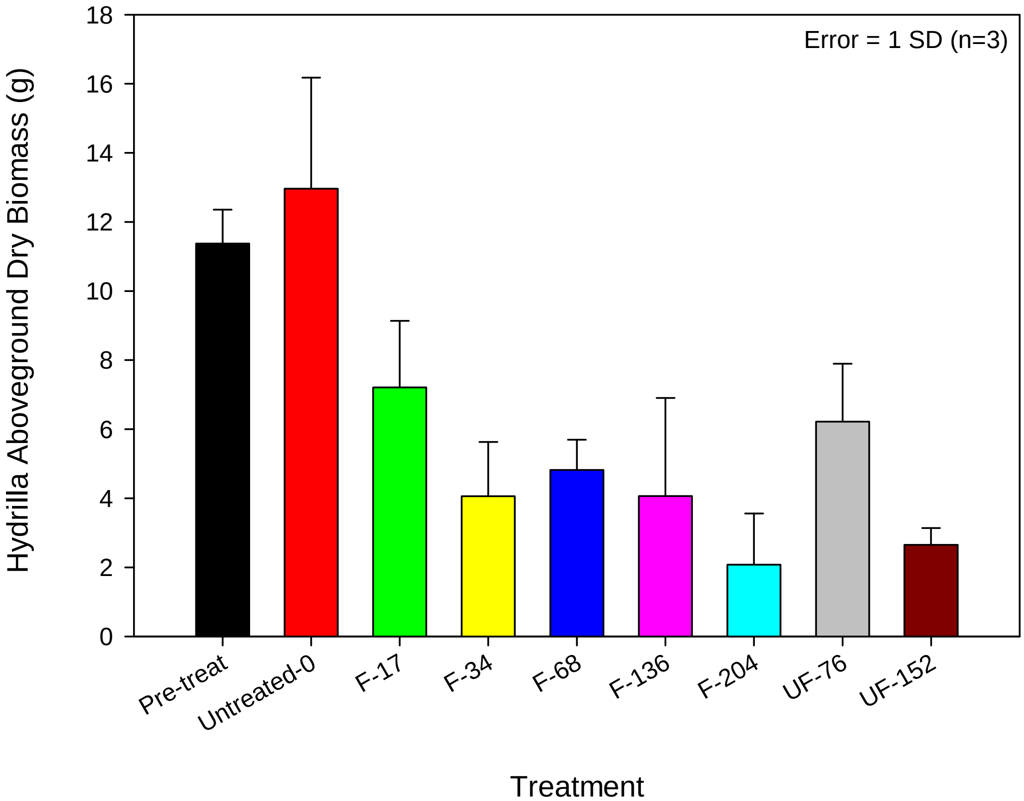

Figure 10. Bioefficacy of dried *M. terrestris* microsclerotia preparations after 3-7 months storage at 4 ºC.