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RESEARCH ARTICLE

The impact of temperature on the production and fitness of microsclerotia of the fungal bioherbicide *Mycoleptodiscus terrestris*[1]

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The impact of growth temperature was evaluated for the fungal plant pathogen *Mycoleptodiscus terrestris* over a range of temperatures (20–36°C). The effect of temperature on biomass accumulation, colony forming units (cfu), and microsclerotia production was determined. Culture temperatures of 24–30°C produced significantly higher biomass accumulations and 20–24°C resulted in a significantly higher cfu. The growth of *M. terrestris* was greatly reduced at temperatures above 30°C and was absent at 36°C. The highest microsclerotia concentrations were produced over a wide range of temperatures (20–30°C). These data suggest that a growth temperature of 24°C would optimize the parameters evaluated in this study. In addition to growth parameters, we also evaluated the desiccation tolerance and storage stability of air-dried microsclerotial preparations from these cultures during storage at 4°C. During 5 months storage, there was no significant difference in viability for air-dried microsclerotial preparations from cultures grown at 20–30°C (>72% hyphal germination) or in conidia production (sporogenic germination) for air-dried preparations from cultures grown at 20–32°C. When the effect of temperature on germination by air-dried microsclerotial preparations was evaluated, data showed that temperatures of 22–30°C were optimal for hyphal and sporogenic germination. Air-dried microsclerotial preparations did not germinate hyphally at 36°C or sporogenically at 20, 32, 34, or 36°C. These data show that temperature does impact the growth and germination of *M. terrestris* and suggest that water temperature may be a critical environmental consideration for the application of air-dried *M. terrestris* preparations for use in controlling hydrilla.

Keywords: sclerotia; bioherbicide; fermentation; hydrilla; aquatic weed

Introduction

Hydrilla verticillata (L.f.) Royle (hydrilla) is considered one of the three most important aquatic weeds in the world (Soerjani 1986). This invasive plant was introduced into Florida in the 1950s and is now found in lakes, ponds, reservoirs, rivers, and canals across the

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southern United States. Hydrilla 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 (Soerjani 1986; Holm, Doll, Holm, Pancho, and Herberger 1997). Plant infestations can impede navigation, clog drainage or irrigation canals, affect water intake systems, interfere with recreational activities, and disrupt wildlife habitats. Management of hydrilla has primarily been practiced with chemical herbicide applications or mechanical removal (Schmitz, Nelson, Nall, and Schardt 1991). The recent appearance of herbicide resistance in some populations of hydrilla combined with environmental concerns regarding herbicide application in aquatic habitats has heightened interest in alternative management options including biological-based control methods (Richardson 2008).

Inundation biocontrol (Harley and Forno 1992; Auld and Morin 1995; Eilenberg, Hajek, and Lomer 2001) of hydrilla with indigenous fungal pathogens of aquatic weeds has been evaluated as a potential control tool since the early 1970s (Charudattan 1973, 2001; Sorsa, Nordheim, and Andrews 1988; Shearer and Grodowitz 2010). Surveys of hydrilla populations in southern United States resulted in the isolation of various indigenous fungal pathogens of hydrilla that were capable of significantly reducing vegetative biomass in greenhouse and field trials (Charudattan 1973, 1981; Joye 1990: Joye and Cofrancesco 1991). A particularly effective fungal pathogen of hydrilla was isolated and originally reported as *Macrophomina phaseolina* (Tassi) Goid (Joye 1990) but later determined to be *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (Shearer 1993). Examination of the host/pathogen relationship at the histological level showed that propagules of *M. terrestris* attached to and penetrated hydrilla tissues within 2 days post inoculation (Joye and Paul 1991). Within 8 days, hydrilla tissues were completely colonized by the pathogen. Subsequent hydrilla collections in Texas resulted in the isolation of a very aggressive strain of *M. terrestris* that demonstrated excellent potential for hydrilla control alone or in combination with chemical herbicides (Netherland and Shearer 1996; Shearer 1997, 1998; Nelson, Shearer, and Netherland 1998; Shearer and Nelson 2002).

Critical constraints to the commercial development of microbial biocontrol agents include the ability of the microorganism to form a stable, infective propagule and the availability of cost-effective method for producing high concentrations of these propagules (Jackson 1997, 2007; Goettel and Roberts 1992; Wraight, Jackson, and De Kock 2001). Numerous fungal plant pathogens, including *M. terrestris*, are capable of producing sclerotia or microsclerotia; i.e., melanized, compact hyphal aggregates that may survive desiccation and serve as the overwintering structure for the fungus (Cooke 1983; Coley-Smith and Cooke 1971; Shearer and Jackson 2006). Sclerotial structures are often formed in the soil or in senescing plant material, germinating when conditions are favorable for infecting their plant host. The stability of dried microsclerotial preparations and their potential to produce infective conidia when conditions are suitable for growth make these propagules a promising fungal form for use as a bioherbicide (Jackson and Schisler 2002). The development of large-scale, liquid culture production methods for fungal microsclerotia of the plant pathogens *Colletotrichum truncatum* and *M. terrestris* and the insect pathogen *Metarhizium anisopliae* have opened the way for the production of stable, dry preparations of these biocontrol agent (Jackson and Schisler 1995; Shearer and Jackson 2006; Jackson and

Jaronski 2009). Conidia produced by germinating microsclerotia of *C. truncatum* and *M. anisopliae* have been shown to be responsible for infecting their respective weed and insect hosts (Schisler and Jackson 1996; Jaronski and Jackson 2008). Microsclerotia of *M. terrestris* formed hyphae (hyphal germination) and conidia (sporogenic germination) that were capable of infecting and killing hydrilla (Shearer and Jackson 2003, 2006; Shearer 2007).

Mycoleptodiscus terrestris has been extensively studied as a bioherbicide for aquatic weeds but remains to be fully developed as a commercial control tool for hydrilla management (Netherland and Shearer 1996; Shearer and Nelson 2002; Nelson and Shearer 2008). Evaluations of hydrilla control with fresh, liquid culture preparations of *M. terrestris* showed a correlation between colony forming units (cfu) and hydrilla infectivity and control with significant reductions in hydrilla biomass using application rates of 100 cfu L^{-1} (Shearer and Nelson 2002). Application rates of 0.25 mg L^{-1} of a dried *M. terrestris* microsclerotia-containing preparation significantly reduce hydrilla biomass with even better control when used in combination with low-dose chemical herbicides (Nelson and Shearer 2005). Research aimed at the commercial scale-up of fermentation methods for producing microsclerotia of *M. terrestris* is underway and this publication includes some of these research results.

Temperature is an environmental factor that may significantly influence culture growth, microsclerotia development, and bioherbicidal activity by *M. terrestris*. Water temperatures can fluctuate dramatically in the fresh-water lakes, reservoirs, ponds, and canals of the southern United States (Bowes, Holaday, and Haller 1979) and has been shown to impact the biocontrol efficacy of other fungal biocontrol agents of hydrilla (Shabana, Cuda, and Charudattan 2003). During the summer, hydrilla grows rapidly toward the water surface accumulating biomass. The hydrilla canopy traps water on the surface where temperatures may exceed 40°C (unpublished results). Many fungal biocontrol agents require conditions conducive to growth in order to infect and kill their pest target and temperature can play an important role in determining their efficacy under field conditions (Hannusch and Boland 1996; Inglis, Duke, Kawchuk, and Goettel 1999).

The production of biomass of *M. terrestris* for use as a biocontrol agent for hydrilla may require that cfu and microsclerotia formation be optimized since both infective hyphae and conidia may be important for infection and control. In order to determine the optimal growth temperature for the mass production of *M. terrestris*, we grew the fungus at various temperatures and measured biomass accumulation, cfu development, and microsclerotia formation. In addition, we dried the biomass from these *M. terrestris* cultures and looked at their storage stability as measured by hyphal and sporogenic germination following rehydration. To identify temperatures for application of *M. terrestris* for hydrilla control that are conducive to fungal growth, we assessed the impact of germination temperature on hyphal and sporogenic germination by air-dried preparations of *M. terrestris* microsclerotia following rehydration and incubation on water agar. To avoid confusion, growth temperature will be used to describe the temperature at which the cultures were grown and germination temperature was used to describe the temperature at which air-dried microsclerotia were rehydrated and incubated on water agar.

Materials and methods

Culture maintenance and inoculum development

Mycoleptodiscus terrestris (Gerd) Ostazeski TX-05 (NRRL 30559) was isolated from infected hydrilla (*Hydrilla verticillata*) in Sheldon Reservoir, Texas and was used throughout these studies. Stock cultures of *M. terrestris* were obtained by growing the fungus on potato dextrose agar (PDA) plates for 3 weeks at room temperatures, cutting the non-sporulated cultures into 1–3-mm^2 agar plugs, and storing these minced cultures in cryovials containing 1 mL of a 10% glycerol solution at -80°C. Inocula for liquid culture microsclerotia production were obtained from PDA plates inoculated with a frozen stock culture of *M. terrestris* and grown at room temperature ($\sim 21^\circ$C) for 2 weeks. Since *M. terrestris* cultures do not sporulate on agar media, minced PDA-grown cultures (1–3-mm^2 agar pieces) were used as inoculum for pre-cultures used in liquid culturing studies.

To obtain hyphal inoculum of *M. terrestris*, 100 mL of pre-culture medium was inoculated with 1–3-mm^2 agar pieces derived from one-quarter plate of a *M. terrestris* culture grown on PDA. Pre-cultures were grown in 250 mL, baffled, Erlenmeyer flasks (#2543-00250, Bellco Glass, Vineland, NJ, USA) at 28°C and 300 rpm in a rotary shaker incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) for 3 days. Our standard microsclerotia production medium was used for *M. terrestris* growth studies and for the production of microsclerotia for use in air-dried microsclerotia germination and conidia production studies.

Media composition and fermentation conditions

The basal salts medium plus trace metals and vitamins used in all liquid cultures of *M. terrestris* contained per liter of de-ionized water: KH_2PO_4, 4 g; $CaCl_2 \cdot 2H_2O$, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 37 mg; $MnSO_4 \cdot H_2O$, 16 mg; $ZnSO_4 \cdot 7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid, 500 μg each; and folic acid, biotin, vitamin B_{12}, 50 μg each. The pre-culture medium for the production of hyphal inoculum of *M. terrestris* contained the basal medium plus 30 g L^{-1} cottonseed meal (Pharmamedia®, Traders protein, Memphis, TN, USA) and 20 g L^{-1} glucose (Difco Laboratories, Detroit, MI, USA). The standard microsclerotia production medium contained the basal medium plus 30 g L^{-1} corn steep liquor powder (Solulys®, Roquette America, Inc., Keokuk, IA, USA) and 60 g L^{-1} glucose. Glucose stock solutions (20%, w/v) were autoclaved separately and added to shake flask cultures prior to culture inoculation. For 100 L fermentor studies, all microsclerotia production medium components were added prior to autoclaving. The medium pH was adjusted to 5.5 prior to autoclaving and pH was uncontrolled during culture growth for both shake flask and fermentor studies.

Growth temperature studies

Growth studies with *M. terrestris* were conducted using 100 mL culture volumes of microsclerotia production medium in 250 mL, baffled, Erlenmeyer flasks grown at various temperatures and 300 rpm in a rotary shaker incubator. A 10-mL, 3-day-old hyphal pre-culture inoculum was used to provide a 10% (v/v) inoculum. The growth temperatures tested were 20–36°C in 2°C increments. Shake flasks containing the

microsclerotia production medium were warmed to the various test temperatures prior to inoculation with the pre-culture inoculum of *M. terrestris*. Cultures of *M. terrestris* were grown at the test temperatures for 7 days. All treatments were replicated 8 times in each experiment and all experiments were repeated at least twice.

Growth parameters

Biomass accumulation, cfu, and microsclerotia measurements were taken at various times during culture growth to evaluate the effect of temperature on these growth parameters. Biomass accumulation was measured on days 0, 1, 2, 3, 4, and 7 (on day 7 for cultures grown at 24, 26, and 28°C) by dry weight determination, as previously described (Shearer and Jackson 2006). Briefly, 1 mL of whole culture broth was collected from culture flasks at various times. The biomass was separated from the spent medium by vacuum filtration onto pre-weighed filter disks (Whatman GF/A, Maidstone, England). Dry weights were determined by drying the biomass and filter disk at 60°C to a constant weight. Colony forming units were determined for each treatment by streaking 1 mL of an appropriate dilution (dilution resulting in 30–300 visible colonies per plate) of each replicate sample onto Petri plates containing Martin's agar, a medium selective for *M. terrestris* (as described in Baruch and Stack 1990). The dilution plates were incubated for 24–48 h at 28°C and examined using a stereo microscope (Olympus SZH10) to determine the total number of colonies forming on each plate. Colony forming units were evaluated in fermentation broth obtained from cultures on days 0, 1, 2, 3, 4, and 7 post-inoculation.

Four days post-inoculation, microsclerotia concentrations were measured microscopically, as previously described (Shearer and Jackson 2006). Briefly, 100 μL of an appropriate dilution of culture broth was placed on a glass slide, overlaid with a large coverslip (24 × 50 mm, #12-545-F, Fisher Scientific) and the total number of microsclerotia in the 100-μL sample counted microscopically (Olympus BX60 light microscope). Only well formed microsclerotia (200–600 μm) were counted. During sampling, microsclerotial suspensions were constantly vortexed to ensure sample homogeneity. For all analyses, two samples were taken from each treatment, all treatments were replicated 8 times in each experiment, and all experiments were repeated at least twice.

Viability and conidia production by air-dried preparations

For storage stability studies, air-dried microsclerotial preparations of *M. terrestris* were obtained from the shake flask cultures grown at temperatures of 20–36°C. The microsclerotia-containing *M. terrestris* biomass was separated from the culture broth by adding 3% (w/v) diatomaceous earth to the whole culture and dewatering the mixture using vacuum filtration. The fermentation broth – diatomaceous earth mixture was poured into a Buchner funnel with an 11-μm filter (Whatman, Grade 1) and vacuum filtered. Diatomaceous earth was added to whole cultures of *M. terrestris* to aid filtering. The wet filter cake was ground to a fine particle size using short pulses from a food processor, thinly layered in a shallow pan, and air-dried overnight in a laboratory drying chamber to less than 4% moisture (Jackson and Payne 2007). The drying chamber was designed to pass a gentle stream of air over the wet, granulated, filter cake under controlled humidity conditions. The moisture content

was determined using a moisture analyzer (MARK II®, Denver Instruments, Tempe, AZ, USA) and is reported as (wet weight minus dry weight)/wet weight × 100. The air-dried microsclerotia-containing diatomaceous earth preparations were vacuum-packaged in polyethylene bags and stored at 4°C.

For storage stability studies with air-dried microsclerotia preparations, 25 mg of the air-dried microsclerotial preparation was sprinkled onto water agar plates that were then incubated at 28°C. Microsclerotia viability was determined by measuring hyphal germination by microsclerotial granules after 24 h incubation. The first 100 granules of the microsclerotial preparation found on each water agar plate were examined with a stereo microscope (Olympus SZH10) and counted as germinated whenever hyphal growth was observed. Results were presented as percent germination. These same plates were incubated for an additional 7 days at 28°C to allow for conidia production. Conidia production was measured by counting the conidia produced on each plate. Briefly, each plate was flooded with 10 mL sterile de-ionized water and the surface scraped with a plastic 10-μL loop (#22-363-600, Fisher Scientific, Pittsburgh, PA, USA) to dislodge conidia. The conidial suspension was pipetted from the rinsed plate and the suspension volume measured. The conidia concentration of each suspension was determined microscopically with a hemacytometer. Conidia production g^{-1} air-dried microsclerotial preparation was calculated and used for comparison between air-dried microsclerotial preparations from cultures grown at different temperatures. Two samples from each treatment were analyzed, all treatments were replicated 6 times, and all experiments were repeated at least twice.

Germination temperature studies with air-dried microsclerotia

The air-dried microsclerotial preparations of *M. terrestris* used to evaluate the effect of germination temperature on hyphal or sporogenic germination were produced in a baffled, 100 L fermentor (D100, B. Braun, Allentown, PA, USA). Our standard microsclerotia production medium plus 10 mL (0.01%, v/v) anti-foam (#204, Sigma Chemical, St Louis, MO, USA) was used for the 100-L fermentation of *M. terrestris*. All medium components were added to the fermentor prior to autoclaving and pH was adjusted to 5.5 and uncontrolled throughout the fermentation. Controlled fermentation conditions were: temperature, 28°C; agitation, 350 rpm using three, 15 cm diameter Rushton impellors; and airflow, 50 L min^{-1} sterile air. The fermentor was inoculated with a 3-L, 3-day-old hyphal pre-culture. The hyphal pre-culture of *M. terrestris* was obtained from nine, 400-mL cultures grown in 1-L baffled, Erlenmeyer flasks at 28°C and 300 rpm in a rotary shaker incubator. These flasks were inoculated with minced, 1–3-mm^2 agar pieces from *M. terrestris* cultures grown on PDA plates, as previously described. The *M. terrestris* microsclerotia fermentation was harvested after 4 days culture growth. For quality assurance, the fermentation broth was streaked onto nutrient agar plates, incubated at 30°C for 48 h and visually evaluated for possible microbial contamination.

The microsclerotia-containing *M. terrestris* biomass produced in the 100-L fermentor was separated from the fermentation broth using a rotary drum vacuum filter ('mini-filter', Komline-Sanderson, Brampton, ON, Canada). The rotary drum vacuum filter was pre-coated with 4 kg of diatomaceous earth (Hyflo Super Cell®, Celite Corporation, Lompoc, CA, USA) to produce a 2.5-cm filter bed on the rotary drum surface. As the diatomaceous earth-coated drum rotated through the

M. terrestris fermentation broth, the spent media was pulled by vacuum through the diatomaceous earth filter and the fungal biomass accumulated on the filter surface. Biomass of *M. terrestris* was separated from the diatomaceous earth filter bed using a stainless steel knife that was programmed to move toward the center of the rotary drum filter at the rate of 127 μm per drum revolution and the drum speed was set at 1 revolution min^{-1}. The wet filter cake of *M. terrestris* microsclerotia-containing biomass obtained from the rotary vacuum filter was ~75% moisture and was granulated using a conical mill (Quadro® Comil® U5, Quadro Engineering, Waterloo, ON, Canada) with a 6.4-mm screen and a rotor speed of 2000 rpm.

This wet granulate of *M. terrestris* microsclerotia-containing biomass was placed in one-quarter sheet, aluminum baking trays (650 × 450 × 30 mm) at a depth of less than 10 mm. Trays were stacked in a baker's rack and baker's rack placed in a modified proofer oven (Model No. RPI-1324, Traulsen Corp., Ft. Worth, TX, USA). The proofer oven had been modified so that gentle airflow was directed over each tray containing the wet *M. terrestris* granulate. These preparations were air-dried overnight at room temperature. When the filter cake of *M. terrestris* biomass was approximately 30% moisture (18 h), the particle size was further reduced to ~250 μm following two passes through the conical mill; one pass using a 2.4-mm screen followed by another pass using a 1.4-mm screen. Once air-dried to less than 4% moisture (~24 h total drying time), the microsclerotia-containing diatomaceous earth preparations were vacuum-packaged in polyethylene bags and stored at 4°C. The moisture content was determined using a moisture analyzer (MARK II®, Denver Instruments) and is calculated as (wet weight minus dry weight)/wet weight × 100.

For germination temperature studies with air-dried microsclerotia preparations, 25 mg of the air-dried microsclerotial preparation was sprinkled onto water agar plates that had been pre-incubated overnight at the temperature to be tested. The temperatures tested were 20–36°C in 2°C increments. Microsclerotia viability and conidia production were measured as previously described in the Growth Temperature Studies section. Two samples from each treatment were analyzed, all treatments were replicated 6 times, and all experiments were repeated at least twice.

Statistical analyses

A General Linear Model (GLM) approach was used to model dependent variables: biomass accumulation, cfu, microsclerotia concentration, and hyphal and sporogenic germination at various germination temperatures. Weighted linear regressions were used to model mean values of the dependent variables to accommodate multiple observations at a single temperature with the weighting equal to 1/variance of the replications. All equations were linear forms (not necessarily the same equations), whose best fits were determined by degrees of freedom adjusted R^2 values, significant parameter estimates ($P \leq 0.05$), and a significant model *F*-statistic ($p \leq 0.01$). Confidence intervals at the 95% α-level were used to compare predicted mean estimates of each dependent variable between temperatures where non-overlap of the 95% confidence intervals (CI) denotes significant differences.

Mean values were compared for hyphal and sporogenic germination by air-dried microsclerotia preparations of *M. terrestris* incubated at 28°C on water agar by ANOVA with Tukey–Kramer mean separation tests ($P \leq 0.05$) using JMP® (SAS, Cary, NC, USA). Data on biomass accumulation and cfu concentrations by cultures

of *M. terrestris* grown at different temperatures and sampled over time are presented as mean values with standard error bars.

Results

Growth temperature studies

Growth parameters

For *M. terrestris* cultures grown at temperatures between 24 and 34°C, biomass accumulation increased progressively for the 4 days sampled (Figure 1). Biomass concentrations peaked at day 3 for cultures grown at 20–22°C and there was no significant increase in fungal biomass over the 4 days sampled (statistical analysis not shown) for cultures grown at 36°C. While a statistically significant increase in biomass was noted for cultures grown for 7 days (growth temperatures of 24, 26, and 28°C), the biomass accumulation was too small to justify the additional fermentation time (Figure 1). A comparison of mean values for biomass accumulation after 4 days growth for all growth temperatures tested showed that cultures grown at 24–30°C produced significantly higher amounts of biomass (Table 1).

Cultures of *M. terrestris* grown at 20–32°C produced cfu increases through the first 4 days of growth (Figure 2). After 7 days growth, cfu continued to increase in cultures grown at 20–26°C and decreased in cultures grown at 28–36°C. Colony forming unit concentrations decreased daily for *M. terrestris* cultures grown at 34–36°C (Figure 2). When mean values for cfu counts in 4-day-old cultures of *M. terrestris* were compared,

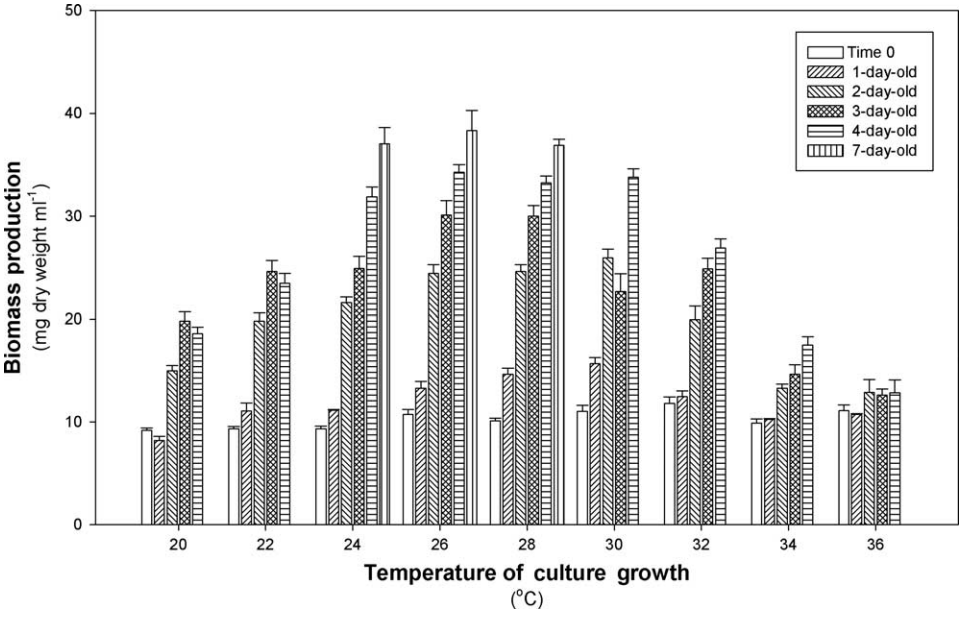

Figure 1. Comparison of biomass accumulation by cultures of *Mycoleptodiscus terrestris* grown in submerged culture at various temperatures in a rotary shaker incubator at 300 rpm. Error bars represent the standard error of the mean value.

Table 1. Comparison of mean values for biomass accumulation, colony forming units, and microsclerotia concentrations for 4-day-old *Mycoleptodiscus terrestris* cultures grown at various temperatures in a rotary shaker incubator at 300 rpm.

Growth temperature (°C)	Biomass (mg mL^{-1})	Colony forming units mL^{-1}	Microsclerotia mL^{-1}
20	18.6 d[1]	1.0×10^7 ab	9.0×10^3 abc
22	23.5 c	1.7×10^7 a	1.2×10^4 a
24	31.9 ab	1.8×10^7 a	1.8×10^4 a
26	34.3 a	1.2×10^6 b	2.1×10^4 a
28	33.2 a	9.6×10^5 b	2.2×10^4 a
30	33.8 a	1.0×10^6 b	1.6×10^4 a
32	26.9 bc	3.5×10^5 b	5.6×10^3 b
34	17.5 d	1.2×10^3 b	3.0×10^3 c
36	12.8 e	1.9×10^2 b	1.6×10^{3c}e
Mean values, post inoculation (time 0)	10.3	9.4×10^4	2.0×10^3

[1]Mean values in columns followed by different letters are significantly different based on non-overlap of 95% CIs of predicted mean values from weighted regression equations.

growth temperatures of 20–24°C resulted in significantly higher cfu ($1.7–1.8 \times 10^7$ mL^{-1}) when compared to cfu values for the other temperatures tested (Table 1).

Cultures grown at 20–30°C produced similarly high microsclerotia concentrations (Table 1). A growth temperature of 36°C proved deleterious for microsclerotia production, yielding a microsclerotium concentration similar to that provided by the inoculum, 1.6×10^3 microsclerotia mL^{-1} (Table 1). A comparison of microsclerotia concentrations in 7- and 4-day-old cultures of *M. terrestris* grown at 24, 26, and 28°C showed no significant difference in mean microsclerotia concentration (data not

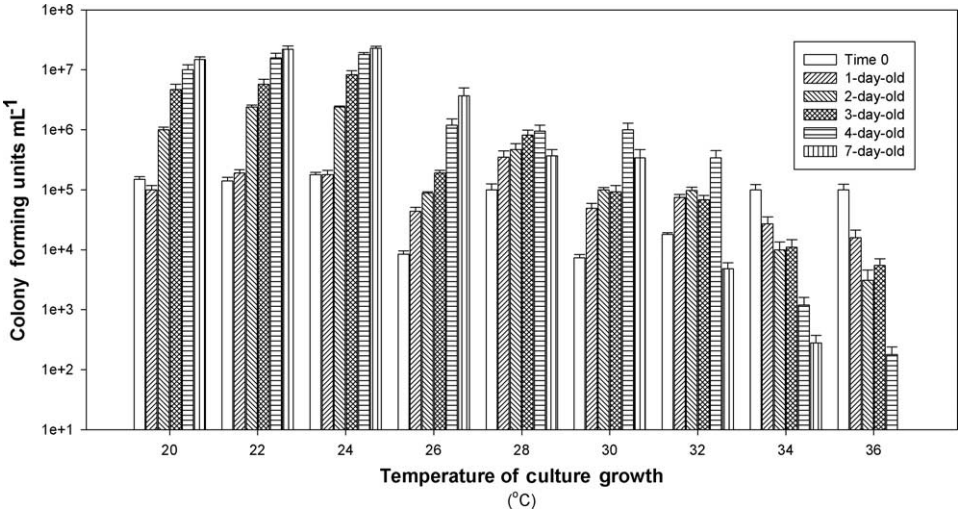

Figure 2. Comparison of colony forming unit development in cultures of *Mycoleptodiscus terrestris* grown in submerged culture at various temperatures in a rotary shaker incubator at 300 rpm. Error bars represent the standard error of the mean value.

shown). Since 4-day-old cultures provided an optimum of biomass and microsclerotia yield at a minimum of fermentation time, the air-dried microsclerotial preparations from *M. terrestris* were derived from 4-day-old cultures.

Viability and conidia production by air-dried preparations

The mean values for viability (hyphal germination) by air-dried microsclerotial granules produced from cultures grown at a wide range of temperatures (20–30°C) were generally not significantly different over the 5 months storage period (Table 2). The hyphal germination rates for microsclerotia from cultures grown at 26–30°C were always over 95% during this period. Under the conditions of this study, microsclerotial granules from cultures grown at 36°C showed virtually no hyphal germination after 24 h incubation at 28°C (Table 2).

All air-dried microsclerotial preparations of *M. terrestris*, regardless of growth temperature, produced conidia following 8 days incubation at 28°C on water agar (Table 3). Microsclerotia preparations from cultures grown at 36°C did not germinate hyphally after 24 h incubation but remained viable and produced more than 10^7 conidia g^{-1} air-dried microsclerotial preparation after 8 days incubation, even after 5 months storage (Table 3). While all microsclerotial preparations from cultures grown at 20–34°C produced conidia concentrations that were in the highest statistical groupings up to 2 months storage, only microsclerotia from cultures incubated at 20–28°C produced conidia concentrations in the highest grouping after 5 months storage.

Germination temperature studies with air-dried microsclerotia

Hyphal germination rate was highest when air-dried microsclerotia were plated and incubated on water agar at 22–30°C (Figure 3). Incubation temperatures of

Table 2. Comparison of hyphal germination by air-dried microsclerotial granules derived from cultures of *Mycoleptodiscus terrestris* grown at various incubation temperatures and stored at 4°C under vacuum.

	Viability (% Germination)					
Culture temperature	0 month	1 month	2 months	3 months	4 months	5 months
20	76.3 a–d[1]	80.9a–d	75.4 a–c	56.1 a–d	50.3 ef	75.3 a–d
22	73.9 a–d	67.3 a–c	70.2 a–c	65.4 a–c	60.4 c–e	72.9 bc
24	75.5 bc	87.5 bc	89.9 b	85.3 ab	76.2 bc	90.3 ab
26	99.5 a–c	99.3 ab	96.7 ab	98.1 ab	99.7 ab	98.2 a
28	99.9 a	99.2 a	96.0 a	98.8 a	99.9 a	95.5 a
30	99.6 ab	99.0 ab	95.7 ab	99.0 ab	99.5 b	94.9 ab
32	97.5 c	94.8 c	92.7 b	96.6 b	80.6 d	73.8 c
34	61.0 d	55.9 d	46.5 c	61.9 c	31.0 f	27.4 d
36	0.9 e	0.1 e	0.1 d	0.3 d	0.3 g	0.0 e

Dried microsclerotial granules rehydrated on water agar and hyphal germination measured after 24 h incubation at 28°C.

[1]Mean values in columns followed by different letters are significantly different based on non-overlap of 95% CIs of predicted mean values from weighted regression equations.

Table 3. Comparison of sporogenic germination by air-dried microsclerotial granules derived from cultures of *Mycoleptodiscus terrestris* grown at various incubation temperatures and stored at 4°C under vacuum.

	Conidia production (conidia produced g^{-1} dried microsclerotial preparation $\times 10^7$)					
Culture temperature	0 month	1 month	2 months	3 months	4 months	5 months
20	11.4 a[1]	11.5 ab	13.0 abc	11.6 bcd	10.4 ab	15.6 a
22	11.4 a	17.3 a	14.8 ab	10.5 cd	10.3 a	15.2 a
24	11.6 a	11.1 ab	16.5 a	12.5 abc	15.7 a	19.3 ab
26	10.1 a	11.0 bc	16.4 a	16.1 a	12.7 a	7.8 ab
28	13.5 a	13.8 ab	19.0 a	20.5 a	12.7 a	9.7 abc
30	9.7 a	12.7 ab	18.7 ab	16.4 ab	10.7 a	8.0 bc
32	15.2 a	17.6 ab	22.1 ab	13.4 bc	17.8 ab	12.5 cd
34	8.6 a	13.2 ab	9.8 bc	6.4 d	7.8 b	6.1 de
36	4.8 b	6.3 c	3.8 c	1.6 e	1.1 c	1.8 e

Conidia production measured after dried microsclerotial granules were rehydrated on water agar and incubated for eight days at 28°C.
[1]Mean values in columns followed by different letters are significantly different based on non-overlap of 95% CIs of predicted mean values from weighted regression equations.

20 or 32°C supported hyphal germination rates of 70 and 55%, respectively. No hyphal germination was observed after microsclerotial preparations were incubated 24 h at 36°C.

Sporogenic germination (conidia production) by air-dried microsclerotial preparations was even more restricted by germination temperature with conidia production being absent when cultures were incubated at 20, 32, 34, or 36°C (Figure 3). The highest concentrations of conidia were produced by air-dried microsclerotial preparations incubated at a germination temperature of 24°C (Figure 3).

Discussion

One of the challenges of developing a fungal biocontrol agent with multiple morphologies is identifying the most efficacious fungal propagule and the formation of these propagules during the fermentation process. Microsclerotia are the desired propagule for dried *M. terrestris* preparations due to their desiccation tolerance and storage stability while whole-culture, liquid preparations may benefit from the higher number of infective propagules in cfu-rich fermentations (Nelson and Shearer 2005; Shearer and Jackson 2006). Using liquid culture fermentation, growth temperature was shown to significantly influence the characteristics of cultures of *M. terrestris*. A growth temperature of 24°C resulted in the highest levels of all the measured parameters; biomass accumulation, cfu, and microsclerotia (Table 1) and produced biomass with excellent desiccation tolerance and storage stability (Tables 2 and 3). This information is of critical importance for guiding mass production of *M. terrestris* for hydrilla control.

Microsclerotia may also have value for use in liquid preparations of this biocontrol agent for hydrilla control due to their potential to tolerate temperature fluctuations found in field conditions. The *M. terrestris* pre-culture inoculum used for

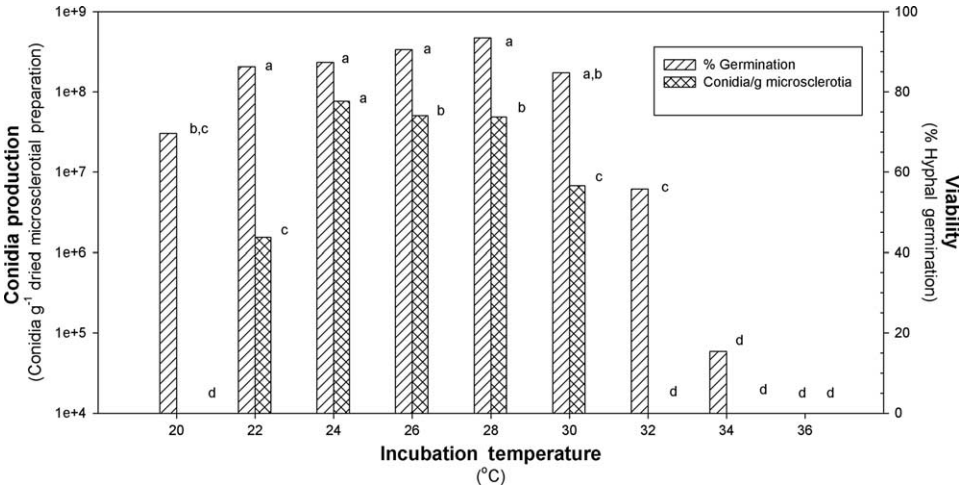

Figure 3. Comparison of hyphal and sporogenic germination rates for air-dried microsclerotial preparations of *Mycoleptodiscus terrestris* incubated at various temperatures on water agar. Mean values in columns followed by different letters are significantly different based on non-overlap of 95% CI of predicted mean values from weighted regression equations.

incubation temperature studies contained sufficient microsclerotia to produce cultures with $\sim 2.0 \times 10^3$ microsclerotia mL^{-1} at the start of incubation (Table 1). While cultures grown at 36°C showed no increase in biomass accumulation or microsclerotia concentration and a 450-fold decrease in cfu, the air-dried preparations from these cultures remained viable and produced conidia *via* sporogenic germination after rehydration and incubation at 28°C (4.8×10^7 conidia g^{-1} dry microsclerotial preparation after drying and 1.8×10^7 conidia g^{-1} after 5 months storage, Table 3). The ability of non-dried microsclerotia of *M. terrestris* to survive for 4 days at 36°C, a temperature that inhibited hyphal or sporogenic germination by air dried microsclerotial preparations (Figure 3), suggests that these propagules may be capable of surviving the higher temperatures occurring in topped-out hydrilla mats. Additional testing is necessary to evaluate the efficacy and persistence of fresh and air-dried microsclerotia of *M. terrestris* when exposed to periods of high temperature.

For commercial use as a living biological control agent, the shelf-life of air-dried microsclerotial preparations of *M. terrestris* must be considered when optimizing production parameters such as temperature. Tables 2 and 3 showed that the temperature used for producing biomass of *M. terrestris* influenced the hyphal and sporogenic germination of the dried microsclerotial preparation. Following rehydration, rapid hyphal germination by dried microsclerotial preparations of *M. terrestris* may enhance their biocontrol efficacy by allowing faster attachment and penetration of hydrilla. In this study, a wide range of growth temperatures (20–30°C) produced air-dried microsclerotial preparations that had similar high hyphal germination rates with cultures grown at 26–30°C maintaining germination rates of more than 95% throughout the 5 months storage period at 4°C (Table 2).

The ability of dried microsclerotial preparations to produce conidia as a secondary inoculum for infecting hydrilla would also seem to be a positive characteristic for enhancing their biocontrol efficacy against hydrilla. Studies have shown that

these conidia infect hydrilla although their correlation to improved biocontrol of hydrilla has not been conclusively demonstrated (unpublished data). All the air-dried microsclerotial preparations from cultures tested in this study produced some microsclerotia even after 5 months storage (Table 3). The microsclerotia present in the culture inoculum were likely responsible for conidia production for cultures of *M. terrestris* grown at 34–36°C as growth and microsclerotia production were minimal at these temperatures. These microsclerotia did not germinate hyphally in 24 h but were still viable and produced conidia after 8 days incubation. Additional studies are needed to document the significance of conidia production as a secondary source of inoculum for enhanced hydrilla infection and control by *M. terrestris*.

The evaluation of hyphal and sporogenic germination by air-dried microsclerotia following rehydration and incubation at various temperatures is informative from a product use standpoint. Hyphal germination by air-dried microsclerotial preparations occurred when they were rehydrated and incubated at 20–34°C and conidia production at 22–30°C (Figure 3). These results suggest that water temperature may significantly affect the ability of *M. terrestris* to grow and sporulate, requirements for infecting and killing hydrilla. Previous studies by Shabana et al. (2003) showed that temperatures between 20 and 30°C resulted in significantly more hydrilla damage by the bioherbicidal fungus *Fusarium culmorum* when compared to treatment at water temperatures of 15 or 35°C. Since water temperatures in impoundments infested by hydrilla can vary significantly depending on the time of year and on the growth status of the hydrilla, understanding the physiological response of *M. terrestris* to these different temperatures is critical to identifying optimal times for application of *M. terrestris* for hydrilla control.

Our studies with *M. terrestris* have shown the importance of temperature in regard to the production and activity of the fungal bioherbicide *M. terrestris*. This information will be useful in developing production strategies based on how the final product will be used, wet or dried, and on how long the dried *M. terrestris* product will be stored. Maximal fungal activity occurred when air-dried microsclerotia were incubated at 22–32°C although *M. terrestris* microsclerotial preparations survived temperatures as high as 36°C for extended periods of time. Studies involving plant inoculations of microsclerotia of *M. terrestris* are needed to confirm the importance of water temperature and to determine appropriate conditions for the use of this mycoherbicide in the field.

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References

Auld, B.A., and Morin, L. (1995), 'Constraints in the Development of Bioherbicides', *Weed Technology*, 9, 638–652.

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

Bowes, G., Holaday, A.S., and Haller, W.T. (1979), 'Seasonal Variation in the Biomass, Tuber Density, and Photosynthetic Metabolism of Hydrilla in Three Florida Lakes', *Journal of Aquatic Plant Management*, 17, 61–65.

Charudattan, R. (1973), 'Pathogenicity of Fungi and Bacteria from India to Hydrilla and Waterhyacinth', *Hyacinth Control Journal*, 11, 44–48.

Charudattan, R. (1981), 'Method and Composition for Controlling Hydrilla', United States Patent No. 4,263,036.

Charudattan, R. (2001), 'Biological Control of Weeds by Means of Plant Pathogens: Significance for Integrated Weed Management in Modern Agro-ecology', *BioControl*, 46, 229–260.

Cooke, R. (1983), 'Morphogenesis of Sclerotia', in *Fungal Differentiation: A Contemporary Synthesis*, ed. J.E. Smith, New York, NY: Marcel Dekker, Inc., pp. 397–418.

Coley-Smith, J.R., and Cooke, R.C. (1971), 'Survival and Germination of Fungal Sclerotia', in *Annual Review of Phytopathology*, eds. J.G. Horsfall, K.F. Baker and G.A. Zentmyer, Palo Alto, CA: Annual Reviews Inc., pp. 65–92.

Eilenberg, J., Hajek, A., and Lomer, C. (2001), 'Suggestions for Unifying the Terminology in Biological Control', *BioControl*, 46, 387–400.

Goettel, M.S., and Roberts, D.W. (1992), 'Mass Production, Formulation and Field Application of Entomopathogenic Fungi', in *Biological Control of Locusts and Grasshoppers*, eds. C.J. Lomer and C. Prior, Wallingford, UK: CAB International, pp. 230–238.

Hannusch, D.J., and Boland, G.J. (1996), 'Interaction of Air Temperature, Relative Humidity and Biological Control Agents on Grey Mold of Bean', *European Journal of Plant Pathology*, 102, 133–142.

Harley, K.L.S., and Forno, I.W. (1992), *Biological Control of Weeds: A Handbook for Practitioners and Students*, Melbourne, Australia: Inkata Press.

Holm, L., Doll, J., Holm, E., Pancho, J., and Herberger, J. (1997), *World Weeds: Natural Histories and Distribution*, New York, NY: John Wiley & Sons, Inc.

Inglis, C.D., Duke, G.M., Kawchuk, L.M., and Goettel, M.S. (1999), 'Influence of Oscillating Temperatures on the Competitive Infection and Colonization of the Migratory Grasshopper by *Beauveria bassiana* and *Metarhizium flavoviride*', *Biological Control*, 14, 111–120.

Jackson, M.A. (1997), 'Optimizing Nutritional Conditions for the Liquid Culture Production of Effective Fungal Biological Control Agents', *Journal of Industrial Microbiology and Biotechnology*, 19, 180–187.

Jackson, M.A. (2007), 'The Biotechnology of Producing and Stabilizing Living, Microbial Biological Control Agents for Insect and Weed Control', in *Biocatalysis and Biotechnology: Functional Foods and Industrial Products*, eds. C.T. Hou and F.J. Shaw, Boca Raton, FL: CRC Press, pp. 533–543.

Jackson, M.A., and Jaronski, S.T. (2009), 'Production of Microsclerotia of the Fungal Entomopathogen *Metarhizium anisopliae* and Their Potential Use as a Biocontrol Agent For soil-inhabiting Insects', *Mycological Research*, 113, 842–850.

Jackson, M.A., and Payne, A.R. (2007), 'Evaluation of Desiccation Tolerance of Blastospores of *Paecilomyces fumosoroseus* (Dueteromycotina: Hyphomycetes) Using a Lab-scale, Air-drying Chamber with Controlled Humidity', *Biocontrol Science and Technology*, 17, 709–719.

Jackson, M.A., and Schisler, D.A. (1995), 'Liquid Culture Production of Microsclerotia of *Colletotrichum truncatum* for Use as Bioherbicidal Propagules', *Mycological Research*, 99, 879–884.

Jackson, M.A., and Schisler, D.A. (2002), 'Selecting Fungal Biocontrol Agents Amenable to Production by Liquid Culture Fermentation', in Proceedings of the 7th Biocontrol Working Group Meeting, *Influence of A-Biotic and Biotic Factors on Biocontrol Agents*, eds. Y. Elad, J. Kohl and D. Shtienberg, *IOBC WPRS Bulletin*, pp. 387–391.

Jaronski, S.T., and Jackson, M.A. (2008), 'Efficacy of *Metarhizium anisopliae* Microsclerotia Granules', *Biocontrol Science and Technology*, 18, 849–863.

Joye, G.F. (1990), 'Biocontrol of Hydrilla with the Endemic Fungus *Macrophomina phaseolina*', *Plant Disease*, 74, 1035–1036.

Joye, G.R., and Cofrancesco Jr, A.F. (1991), 'Studies on the Use of Fungal Plant Pathogens for Control of *Hydrilla verticillata* (L.f.) Royle', Technical Report A-91-4, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Joye, G.F., and Paul, R.N. (1991), 'Histology of Infection of *Hydrilla verticillata* by *Macrophomina phaseolina*', *Weed Science*, 40, 288–295.

Netherland, M.D., and Shearer, J.F. (1996), 'Integrated Use of Fluridone and a Fungal Pathogen for Control of Hydrilla', *Journal of Aquatic Plant Management*, 33, 4–8.

Nelson, L.S., and Shearer, J.F. (2005), '2,4-D and *Mycoleptodiscus terrestris* for Control of Eurasian Watermilfoil', *Journal of Aquatic Plant Management*, 43, 29–34.

Nelson, L.S., and Shearer, J.F. (2008), 'Evaluation of Triclopyr and *Mycoleptodiscus terrestris* for Control of Eurasian Watermilfoil (*Myriophyllum spicatum*)', *Invasive Plant Science and Management*, 1, 337–342.

Nelson, L.S., Shearer, J.F., and Netherland, M.D. (1998), 'Mesocosm Evaluation of Integrated Fluridone-fungal Pathogen Treatment of Four Submersed Plants', *Journal of Aquatic Plant Management*, 36, 73–77.

Richardson, R.J. (2008), 'Aquatic Plant Management and the Impact of Emerging Herbicide Resistance Issues', *Weed Technology*, 22, 8–15.

Schisler, D.A., and Jackson, M.A. (1996), 'Germination of Soil-incorporated Microsclerotia of *Colletotrichum truncatum* and Colonization of Seedlings of the Weed *Sesbania exaltata*', *Canadian Journal of Microbiology*, 42, 1032–1038.

Schmitz, D.C., Nelson, B.V., Nall, L.E., and Schardt, J.D. (1991), 'Exotic Aquatic Plants in Florida: A Historical Perspective and Review of the Present Aquatic Plant Regulation Program', in *Proceedings of the Symposium on Exotic Pest Plants*, 1988, University of Miami, Rosenstiel School of Marine and Atmospheric Science, Miami, FL. Eds. Center, Washington, DC: United States Department of the Interior, National Park Service Document, pp. 303–323.

Shabana, Y.M., Cuda, J.P., and Charudattan, R. (2003), 'Combining Plant Pathogenic Fungi and the Leaf-mining Fly, *Hydrellia pakistanae*, Increases Damage to Hydrilla', *Journal of Aquatic Plant Management*, 41, 76–81.

Shearer, J.F. (1993), 'Biocontrol of Hydrilla and Milfoil using Plant Pathogens', Proceedings of the 27th Annual Meeting, Aquatic Plant Control Research Program. Miscellaneous Paper, A-93-2, US Army Engineer Waterways Experiment Station, Vicksburg, MS, pp. 79–80.

Shearer, J.F. (1997), 'Endemic Pathogen Biocontrol Research on Submersed Macrophytes: Status report 1996', Technical Report A-97-3, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Shearer, J.F. (1998), 'Biological Control of Hydrilla using an Endemic Fungal Pathogen', *Journal of Aquatic Plant Management*, 36, 54–56.

Shearer, J.F. (2007), 'Some Observations Concerning Microsclerotia and Spore Production of *Mycoleptodiscus terrestris* in Culture', *Mycologia*, 99, 88–90.

Shearer, J.F., and Grodowitz, M.J. (2010), 'Effect of Dried *Mycoleptodiscus terrestris* Inoculums on Fluridone-susceptible and Fluridone-resistant Hydrilla Biotypes', APCRP Technical Notes Collection, ERDC/TN APCRP-BC-19. Vicksburg, MS: U.S. Army Engineer research and Development Center. Available from: www.wes.army.mil/el/aqua.

Shearer, J.F., and Jackson, M.A. (2003), 'Mycoherbicidal Compositions and Methods of Preparing and Using the Same', United States patent No. 6,569,807.

Shearer, J.F., and Jackson, M.A. (2006), 'Liquid Culturing of Microsclerotia of *Mycoleptodiscus terrestris*, a Potential Biological Control Agent for the Management of Hydrilla', *Biological Control*, 38, 298–306.

Shearer, J.F., and Nelson, L.S. (2002), 'Integrated use of Endothall and a Fungal Pathogen for Management of the Submersed Aquatic Macrophyte *Hydrilla verticillata*', *Weed Technology*, 16, 224–230.

Soerjani, M. (1986), 'Environmental Consideration in the Novel Approach of Aquatic Vegetation Management', in *Weeds and the Environment in the Tropics*, Proceedings of the 10th Asian-Pacific Weed Science Society Conference, Chi Maing, Thailand, pp. 33–49.

Sorsa, K.K., Nordheim, E.V., and Andrews, J.H. (1988), 'Integrated Control of Eurasian Watermilfoil, *Myriophyllum spicatum*, by a Fungal Pathogen and a Herbicide', *Journal of Aquatic Plant Management*, 26, 12–17.

Wraight, S.P., Jackson, M.A., and De Kock, S.L. (2001), 'Production, Stabilization and Formulation of Fungal Biocontrol Agents', in *Fungi as Biocontrol Agents: Progress, Problems and Potential*, eds. T.M. Butt, C. Jackson and N. Magan, Wallingford, UK: CABI Publishing, pp. 253–288.