

Submerged culture of a mycelial formulation of a bioherbicidal strain of *Myrothecium verrucaria* with mitigated mycotoxin production

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Abstract A mycelial formulation of the fungus *Myrothecium verrucaria* (IMI 361690) containing 0.20% Silwet L-77 surfactant was found to be highly efficacious in controlling the exotic invasive weed kudzu. The mycelium can be rapidly (48–72 h) produced in several media, including an inexpensive soy flour–corn meal medium. Mycelial yields were 2, 10, and 25 g dry weight l⁻¹ in Czapek-Dox, Richard’s V-8, and soy flour–corn meal media, respectively. Scale-up production in soy flour–corn meal medium using laboratory fermenters (10–25 l), resulted in a mycelial formulation that caused 90% mortality of naturally-occurring mature (0.9–1.0 m in height) kudzu within 48 h after application in field experiments. HPLC analyses revealed that the mycelium produced in this liquid culture contained no detectable amounts of the trichothecene mycotoxins roridin A and verrucarins A (limit of detection 2 µg ml⁻¹). This has resulted in a safer, yet effective bioherbicidal product. We anticipate that these findings should improve the probability of EPA registration and subsequent commercial development of this bioherbicide.

Keywords Bioherbicide · Formulation · Fermentation · *Myrothecium verrucaria* · *Pueraria lobata* var. *montana*

Introduction

Myrothecium verrucaria (Alb. & Schwein.) Ditmar:Fr. is a cellulolytic fungus of the class Hyphomycetes. Fungal spores

of *M. verrucaria* (IMI 361690) exhibit biocontrol potential against several weed species, including sicklepod [*Senna obtusifolia* (L.) H.S. Barneby], hemp sesbania [*Sesbania exaltata* (Rydb.) ex Cory] (Walker and Tilley 1997; Anderson and Hallett 2004) and kudzu [*Pueraria lobata* var. *montana* Willd. (Ohwii.)] (Boyette et al. 2002) when formulated with Silwet L-77 surfactant (a silicone-polyether copolymer spray adjuvant, OSi Specialties, Inc., Charlotte, NC, USA). Other strains of this fungus, including *M. verrucaria* (ATCC 18398) applied in an invert emulsion (Connick et al. 1991) also have shown biological control potential against leafy spurge (*Euphorbia maculata* L) (Yang and Jong 1995a, b), and morningglory spp. (*Ipomoea* spp.) (Milhollon et al. 2003).

Although *M. verrucaria* (IMI 361690) can effectively control several weed species, mycotoxin production (e.g. macrocyclic trichothecene verrucarins) (Abbas et al. 2002) has hindered U.S. Environmental Protection Agency (US-EPA) registration of this bioherbicide, and subsequently lead to a lack of industrial interest in developing *M. verrucaria* (IMI 361690) as a commercial bioherbicide. However, a formulation consisting of heat-killed fungal cells from another *M. verrucaria* strain (ATCC 46474) has received US-EPA registration as a biologically-based nematocide (DiTeraTM, Valent BioSciences Corp., Walnut Creek, CA, USA) (Warrior et al. 2000). This product was used for control of root knot nematodes [*Meloidogyna incognita* (Kof & White) Chitwood]. Based on acute toxicology batteries, this product and its formulations were classified as US-EPA Category III (slightly toxic) and Category IV (practically non-toxic) pesticides (Warrior et al. 1999).

The majority of fungi that have been evaluated as bioherbicides utilize fungal conidia (spores) as the active component (Charudattan 2001). However, there are also

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reports of highly effective mycelial bioherbicide formulations (Conway 1976; Boyette et al. 1991; Ghorboni et al. 2000; Elzein and Kroschel 2004). Preliminary experiments (unpublished) in our laboratory demonstrated that liquid culture of *M. verrucaria* (IMI 361690) using soy flour–corn meal medium (Walker 1982) produced mycelium that was bioherbicidally active. Thus, our objectives here were to: (1) evaluate the production, bioherbicidal activity, and trichothecene content of *M. verrucaria* mycelia when grown in several different liquid media; and (2) select the optimal media with respect to mycelial production and low trichothecene content, and use this medium for rapid production of large amounts of mycelial inoculum in liquid fermentation to use in field testing of this bioherbicide.

Materials and methods

Accession, culture, and maintenance of the fungus

A single strain of *M. verrucaria* (IMI 361390) was used throughout these studies. A culture of the fungus, originally isolated from diseased sicklepod (Walker and Tilley 1997), was obtained from H. L. Walker, Department of Biological Sciences, Louisiana Tech. University, Ruston, LA, USA. The fungus was sub-cultured in Petri dishes containing potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA). Stock cultures were stored in twice-sterilized soil (25% water holding capacity) at 4°C (Tuite 1969). The inoculated plates were inverted and placed on open-mesh wire shelves of an incubator (Precision Scientific Inc., Chicago, IL, USA) at 28°C. Twelve-hour photoperiods were provided to induce sporulation (Walker and Tilley 1997) by two-20W cool-white fluorescent lamps positioned in the incubator door. Light intensity at dish level was approximately 200 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetically active radiation (PAR) as measured with a light meter.

Inoculum production

Three different media were tested for mycelial production and toxicological assays: (1) Czapek-Dox (Difco Inc., Detroit, MI, USA); (2) Richard's V-8 (Daniel et al. 1973); and (3) soy flour–corn meal medium (Walker 1982). Three replicates of two-liter Erlenmeyer flasks, each containing 500 ml of medium, were inoculated with a 10 mm diameter PDA disk containing *M. verrucaria* conidia and mycelium. The shake-flasks were incubated in rotary shakers at 185–200 rpm and 28°C for 7 days. Samples (1 ml) were taken from each culture using a sterile pipette and microscopically examined for fungal growth. The mycelia were harvested from the flasks, filtered through tared #40 Whatman filter paper, oven-dried at 80°C for

24 h, and dry weight determinations were conducted. Because soy flour–corn meal medium produced the highest mycelial yields of this fungus in shake flasks, this medium was chosen for scaled-up production in laboratory fermenters (Models MF-214 and CMF-128, New Brunswick Corp., Edison, NJ, USA). Fermentations were conducted at 185–200 rpm and 28°C for 48 h. In HPLC analyses and bioassays for efficacy in the greenhouse and field, the raw fermentation product (liquid, unspent medium, and mycelium) was homogenized in ~3–4 l aliquots using an electric blender (high speed, 3 min, Waring Model CB1043, Springfield, MO, USA). All tests were repeated over time.

HPLC analyses

Homogenized *M. verrucaria* mycelial (10 ml) samples from each culture medium were collected for HPLC analyses for the presence of macrocyclic trichothecene as described (Weaver et al. 2007). Briefly, raw fermentation product (mycelium with unspent medium) was contained in 50 ml conical centrifuge tubes, and shaken for ≥ 2 h at 125 rpm in a 1:1 ratio (culture mass:ethanol volume) of ethanol. The ethanol was decanted, centrifuged at 14,000 RCF for 1 min to remove particulate matter, and 1 ml of the clarified supernatant was transferred to amber glass vials. Twenty μl of each sample were analyzed via a Dionex (Sunnyvale, CA, USA), HPLC system equipped with a Polar Advantage II C18 monolithic column (3 μm , 4.6 \times 50 mm, Dionex) incubated in a thermostated (TCC100 40°C) column oven and a UVD340U photo diode array detector. The mobile phase consisted of (A) water amended with 0.1% acetic acid and (B) acetonitrile amended with 0.1% acetic acid. Binary gradient elution starting conditions were 35% B for 2 min, followed by an increase to 50% B at 4.5 min; 58% at 6 min; 70% at 7.29 min; 80% at 7.84 min; 95% at 8.5 min, holding for 0.5 min before returning to initial conditions. Commercially available roridin A and verrucaridin A (Sigma Chemical Co., St. Louis, MO, USA) were used as standards and absorbance of peak area at 260 nm was used to quantify these mycotoxins by the external standard method. The limit of detection in this system was 2 $\mu\text{g ml}^{-1}$.

Greenhouse bioassays

Kudzu seed (obtained from Adams-Briscoe Seed Co., Jackson, GA, USA) were placed on moistened filter paper contained in Petri dishes, and incubated at 28°C for 3 days in a darkened incubator to induce seed germination. Germinated seeds were planted in 10 cm plastic pots (1 seed per pot) containing a 1:1 commercial potting mix (Jiffy Products of America, Inc., Batavia, IL, USA)/sandy loam

soil combination that was supplemented with a controlled-release (14:14:14, N:P:K) fertilizer (Grace Sierra Horticultural Products, Milpitas, CA, USA) and placed on greenhouse benches. The plants were sub-irrigated daily. Temperatures in the greenhouse ranged from 28 to 32°C with 40% to 60% relative humidity. The photoperiod was approximately 14 h, with 1,600 to 1,800 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR at midday. Seedlings at the second-to-fourth leaf growth stage were inoculated with raw fermentation product containing 0.2% (v/v) Silwet L-77 surfactant. A hand-held pressurized sprayer (SPRAY DOC[®], Model 050P, Gilmour Mfg., Somerset, PA, USA) was used to apply the inoculum until the foliage was fully wetted (ca 300 l ha⁻¹). Following inoculation, the plants were placed directly on greenhouse benches and monitored for disease development. The data presented represent weed mortality recorded 3 days after inoculation. Treated plants were not subjected to a dew treatment. Experimental units consisted of groups of 10 pots, each containing one seedling. The treatments were replicated three times and the experiment was conducted twice. In this experiment and throughout, mortality and dry weight data were transformed with the arc sin transformation prior to analysis. A randomized complete block experimental design was utilized, and data were analyzed using regression analysis and confidence limits (95% level) (Steele et al. 1997).

Field bioassays

Kudzu test plots (9 m²) were established at two different locations, representing different environmental regimes, near Stoneville and Eden, MS, USA in June and August 2006. Kudzu plants were ~0.90–1.0 m tall at treatment. In all tests, raw fermentation product in 0.2% Silwet L-77 surfactant was sprayed at a rate of ~300 l ha⁻¹ using a CO₂ backpack sprayer (R & D Sprayers, Opalouzas, LA, USA). Kudzu mortality was assessed visually using the paired *t*-test, by comparing the percentage of necrotic tissue in the treated plots with the tissue in the paired untreated control plots (Steele et al. 1997). The experiment consisted of four replications and was repeated in time over the growing season.

Results and discussion

Inoculum production

In shake-flasks, maximal mycelium growth occurred in soy flour–corn meal, producing thick vegetative growth after 7 days (Table 1). Microscopic examinations revealed that no sporulation had occurred in any of the media that were tested. There appeared to be no differences in phytotoxicity

Table 1 Production of *Myrothecium verrucaria* mycelium after 7 days in different liquid culture media^a

| Medium | Mycelial yield (g l ⁻¹) ^b |
|---------------------|--|
| Czapek-Dox | 2 (0.3) |
| Richard's V-8 | 10 (4) |
| Soy flour–corn meal | 25 (5) |

^a Values in parentheses represent 1 SEM

^b Mycelial yields based on oven-dried mycelium (24 h, 80°C)

or trichothecene content (HPLC analyses) in fungal mycelia or culture broths in any of the media tested. Therefore, because of lower production expenses and higher mycelial yields, soy flour–corn meal medium was selected for mass production (fermentation) for all subsequent use in greenhouse and field experiments. In laboratory fermenters, mycelial growth occurred much more rapidly than in shake flasks, requiring only 48 h to reach maximal growth in batch production of 10–20 l.

HPLC analyses

Because there were no differences in mycotoxin content between the three media that were tested (data not shown), only data from the soy flour–corn meal media are presented. In both shake flasks and fermentation production, HPLC analysis revealed that macrocyclic trichothecene mycotoxin levels of roridin A and verrucaridin A levels were either eliminated or reduced to undetectable concentrations (Fig. 1). A minor absorbance peak was noted in the fermentation product with a spectrum that is consistent with macrocyclic trichothecenes (Fig. 1), but differed in retention time from the commercial standards of roridin A and verrucaridin A. The identity of this minor peak is being evaluated.

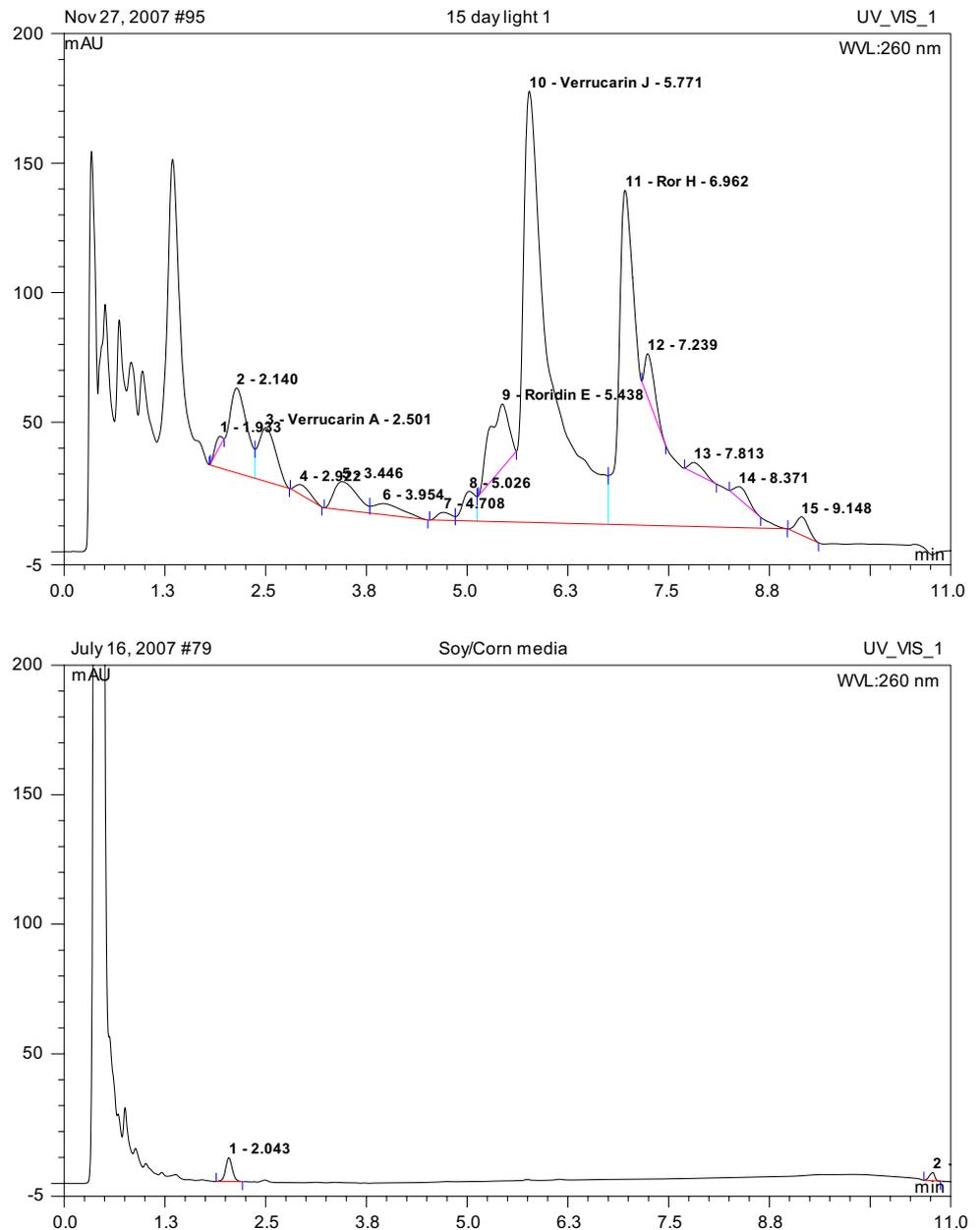
Greenhouse bioassays

Greenhouse experiments revealed that raw *M. verrucaria* fermentation product was highly effective in infecting and killing kudzu. Mortality and dry weight reductions of kudzu occurred at all mycelial concentrations that were tested; and as expected, greater mortality and dry weight reductions occurred at higher mycelial concentrations (Figs. 2 and 3).

Field bioassays

Fields bioassays corroborated our findings in greenhouse experiments. Over 80% of kudzu was controlled within 24 h after inoculation, with maximum mortality rates occurring within 72 h after inoculation. (Figs. 4 and 5).

Fig. 1 HPLC analyses of macrocyclic trichothecenes. Ethanol extract of *Myrothecium verrucaria* grown on PDA (top) and ethanol extract of raw fermentation product containing mycelium (bottom)



Disease symptoms were characterized by rapid necrosis which began to occur within 6 h after inoculation. Because of this rapid necrosis, it is possible that secondary metabolites, such as cellulolytic enzymes, or an unknown phytotoxin(s) are produced by this fungus. Preliminary research has shown that crude enzyme extracts from sonicated *M. verrucaria* mycelium exhibit proteolytic and polysaccharidase activities, as well as hydrolytic activity on fluorescein diacetate, a general substrate for the assay of lipases, esterases, and proteases (Hoagland et al. 2007). Further characterization of these enzymes and their roles in the development of infectivity, necrosis, and mortality of target weeds are subjects of further research.

It is well established that some, and perhaps most strains of *M. verrucaria* produce macrocyclic trichothecene mycotoxins, but Abbas et al. (2001) was not able to detect their presence in *M. verrucaria* (IMI 361390)-infected plants, and Milhollon et al. (2003) found only trace amounts of macrocyclic trichothecenes on a different strain (ATCC 18398) of *M. verrucaria*-infected morningglory.

Myrothecium verrucaria (IMI 361390) has potential to control several invasive weeds such as sicklepod and hemp sesbania (Walker and Tilley 1997), and kudzu, redvine (*Brunnichia ovata* L.), and trumpet creeper (*Campsis radicans* L.) (Boyette et al. 2002, 2006). Because kudzu is a major problem on low-value, non-agronomic lands, and

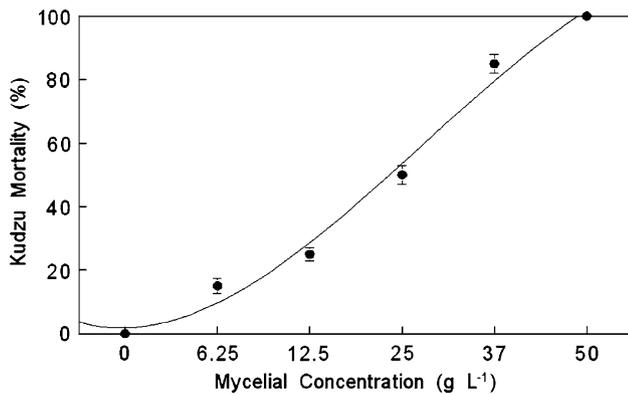


Fig. 2 Effect of *Myrothecium verrucaria* (IMI 361390) mycelial concentrations on kudzu mortality. The best curve fit is a third-degree polynomial described by the equation $Y = 10 - 17.92X + 10.54X^2 - 0.83X^3$ ($R^2 = 0.984$), where X = dry mycelial weight ($g\ L^{-1}$) and Y = kudzu mortality (%)

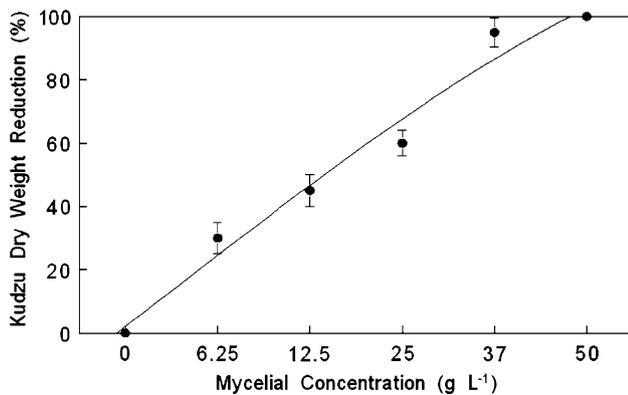


Fig. 3 Effect of *Myrothecium verrucaria* (IMI 361390) mycelial concentrations on kudzu dry weight reduction. The best curve fit is a third-degree polynomial described by the equation $Y = -20X + 21.51X^2 - 0.14X^3$ ($R^2 = 0.982$), where X = dry mycelial weight ($g\ L^{-1}$) and Y = kudzu dry weight reduction (%)

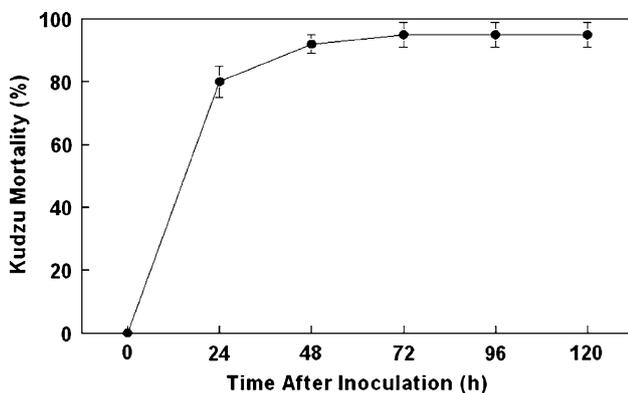


Fig. 4 Biological control of kudzu under field conditions with raw *Myrothecium verrucaria* (IMI 361390) fermentation product. Error bars represent ± 1 SEM



Fig. 5 Kudzu treated with a raw fermentation mycelial product of *Myrothecium verrucaria* (right) and untreated kudzu (left), near Eden MS, USA, August 30, 2006. This photograph was taken approximately 48 h after treatment

traditional weed control options have not been well developed (Miller 1997), a mycotoxin-free formulation of *M. verrucaria* may prove to be an attractive agent for commercial development.

Conclusion

Myrothecium verrucaria mycelium is highly effective for biological control of kudzu. The mycelium can be rapidly produced on inexpensive agricultural products, e.g., soy flour–corn meal medium. Of more importance, macrocyclic trichothecene production in *Myrothecium verrucaria* mycelium is greatly reduced or eliminated, thus providing a safe, highly efficacious bioherbicidal product. These findings may improve the probability of US-EPA registration and commercial development.

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