

Bioherbicidal Efficacy of a *Myrothecium verrucaria*-Sector on Several Plant Species

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Abstract

Comparative studies were conducted on mycelial preparations of the bioherbicide, *Myrothecium verrucaria* (MV) strain IMI 361690 and a recently discovered sector (MV-Sector BSH) of this fungus. The whitish sector was discovered, isolated, grown in pure culture on PDA and found to be a stable, non-spore producing mutant when cultured over several months under conditions that cause circadian sporulation during growth of its MV parent. Application of MV and MV-Sector BSH mycelial preparations to intact plants (hemp sesbania and sicklepod) and leaf discs (kudzu and glyphosate-resistant Palmer amaranth) showed that the sector efficacy was generally equal to, or slightly lower than MV. Bioassays of MV and this sector on seed germination and early growth of sicklepod and hemp sesbania seeds demonstrated that hemp sesbania seeds were slightly more sensitive to the fungus than sicklepod seeds and that the sector bioherbicidal activity was slightly less than that of MV. SDS-PAGE protein profiles of cellular extracts of MV and the sector and their respective culture supernatants showed several differences with respect to quantity and number of certain protein bands. Overall results showed that the isolate was a non-spore producing mutant with phytotoxicity to several weeds (including weeds tolerant or resistant to glyphosate), and that the phytotoxic effects were generally equivalent to those caused by MV treatment. Results of this first report of a non-sporulating MV mutant that suggest additional studies on protein analysis, and an extended weed host range under greenhouse and field conditions are needed in order to further evaluate its possible bioherbicidal potential.

Keywords

Bioherbicide, Glyphosate-Resistance, Palmer Amaranth, Kudzu, Hemp Sesbania, Sicklepod, *Myrothecium verrucaria*, Fungal Sector

1. Introduction

Bioherbicides may offer alternatives to the control of weeds with synthetic compounds including the use of plant pathogens as bioherbicides [1] [2] [3] [4]. Results from our laboratory demonstrated that the bioherbicidal fungus, *Myrothecium verrucaria* (Alb. and Schwein.) Ditmar: Fr. (strain IMI 361690) (MV), can control several weeds from various families [5]-[10]. A substantial portion of this research has been devoted to the potential of MV to control several economically important weeds including, hemp sesbania [*Sesbania herbacea* (Mill.) McVaugh] [11], sicklepod (*Senna obtusifolia*) [12], kudzu (*Pueraria lobata*) [13] [14] [15] and glyphosate-resistant and -susceptible Palmer amaranth (*Amaranthus palmeri* S. Wats.) [16].

Kudzu is an exotic invasive weed in the southeastern US that is difficult to control with current commercial herbicides [17] [18] [19] and this weed has had a devastating impact on forests, rights of ways, etc. [20]. Kudzu, a perennial leguminous vine native to eastern Asia, was introduced into the US in the late 1800's and now occurs from Florida to New York, westward to central Oklahoma and Texas, with heavy infestations in Alabama, Georgia, and Mississippi [21] [22] [23]. In 1993, a Congressional Report cited kudzu as one of the most harmful non-indigenous plants in the US. Some success for its control has been achieved using a bioherbicidal agent, MV. Spore and mycelial formulations of MV tested alone and in combination with glyphosate for control of kudzu under greenhouse and field conditions demonstrated important synergistic actions between herbicide and this bioherbicide on kudzu [13] [15] [24].

Palmer amaranth, originally native to the North American southwest, is an invasive species that has spread rapidly to eastern North America and in some other countries [25]. Now widely distributed in the southeastern United States [26], this major weed has evolved resistance to several herbicides, including triazine, acetolactate-synthase inhibitor, and dinitroaniline herbicides use [27]. Originally it was controlled with glyphosate, but the first glyphosate-resistant Palmer amaranth population was reported in Georgia in 2006 [28]. Its rapid evolved resistance to glyphosate has now resulted in a major epidemic in the southern US and nearly 40 cases of resistance in this weed are now reported [27]. Initial host range studies of MV indicated that some phytotoxicity to redroot pigweed (*Amaranthus retroflexus*) [29], and more recently tests on Palmer amaranth demonstrated bioherbicidal activity on plants that were sensitive or resistant to glyphosate [16].

Hemp sesbania is an aggressive, nodulating legume weed that infests several major crops: rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.) and sunflower (*Helianthus annuus* L.) [30]. It occurs in ditches, on stream banks, fallow areas and waste places in the US coastal plain of Virginia to Florida to Texas [31] and rated as one in the top 10 most troublesome weeds in Arkansas, Louisiana, and Mississippi [32]. Some single or multiple applications of herbicide treatments can provide control of hemp sesbania, but it exhibits some tolerance to glyphosate [33] [34] [35]. MV was shown to be efficacious on hemp sesbania in host range tests [29] and later field studies demonstrated its effectiveness on control of this weed in rice [11].

Sicklepod has been a very problematic weed in several peanut producing states [36]. It has also been reported as a pest in cotton, corn, and soybeans, reduced cotton yield by ~3% per weed per each 9 m row [37], and was classified as one of the world's worst weeds [38]. Full-season interference by sicklepod was shown to reduce peanut yield by 70%, [39]. Various herbicides have been evaluated of control of this weed, and some were highly efficacious [36]. Although generally not currently a major problematic weed, sicklepod was the original host from which MV (strain IMI 361690) was isolated [29] and this weed has been a target of several bioherbicide studies using MV [12] [29].

In early studies with MV, formulations of fungal spores were used as the bioherbicide agent. However, more recent research showed that mycelial formulations of MV (produced via liquid fermentation) were equally efficacious, easier to handle and lacked significant levels of trichothecenes [40]. Very recently, we observed a fungal sector growing on a Petri plate during maintenance culture of MV. Preliminary examination of this spontaneous sector showed it to be a white and non-sporulating mutant (MV-Sector BSH). The objectives of further studies on this sector were to isolate and culture it, and to characterize its efficacy compared with MV-parent. To achieve this, greenhouse and bioassay tests were performed on several weeds with demonstrated susceptibility to MV as outlined above, *i.e.*, hemp sesbania, sicklepod, kudzu and glyphosate-resistant Palmer amaranth.

2. Materials and Methods

2.1. Sources of Seeds and Chemicals

Sicklepod and hemp sesbania seeds were obtained from field-grown plants in a weed nursery at Stoneville, MS. Seeds of Palmer amaranth (glyphosate resistant) were collected from a population in Washington Co., MS and confirmed via bioassays to be resistant to this herbicide [41]. Kudzu seeds were obtained from Adams-Briscoe Seed Co., Jackson, GA 30233, USA. Silwet L-77 (non-ionic surfactant) was procured from OSI Specialties, Inc., Danbury, CT, USA. Potato dextrose agar (PDA), were purchased from Difco Laboratories, Inc. (Detroit, MI). All other chemicals used in these studies were of reagent grade or higher purity.

2.2. MV Source and Culture

MV strain (IMI 361690), originally isolated from sicklepod [29] was used throughout these studies. This strain was cultured in Petri dishes containing PDA from a single spore (MV) or from a fragment of mycelium (MV-Sector BSH). Inoculated plates were then inverted on open-mesh wire shelves and incubated (25°C - 28°C 10 days) in lighted incubators. Photoperiods (12 h) were provided by cool-white fluorescent lamps at an intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation (PAR) as measured with a light meter (LI-COR, Inc., Lincoln, NE 38504). Shake-flask liquid culture was used to produce mycelial products by inoculation of each flask with an 8-mm diameter section from the edge of an actively growing MV culture or the MV-Sector BSH on PDA plates. Sector isolates were stored on PDA slants at 48°C until cultured as de-

scribed above.

2.3. Culture of Plant Seedlings

Seeds of the glyphosate-resistant Palmer amaranth population and kudzu, were planted in a commercial potting mix Jiffy-mix (Jiffy Products of America, Inc., Batavia, IL 60510) contained in 12-cm plastic pots. The pots were placed in large sub-irrigated trays that were placed on greenhouse benches. Greenhouse temperatures ranged from 28°C to 32°C with 50% - 85% relative humidity (RH). The photoperiod was 12 - 14 h with 1600 - 1700 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (PAR). After germination and emergence, the plants were thinned to 2 - 3 per pot. Palmer amaranth was grown to a mature stage (12 - 15 leaves) and kudzu was grown to the vining stage that possessed 6 - 8 trifoliate leaves per plant. Leaves of both of these species were used in the leaf disc bioassay studies. Hemp sesbania and sicklepod seeds were planted in small plastic pots (7-cm) in the same potting mix, and pots were placed in large trays for sub-irrigated watering in the greenhouse under the conditions described above. Hemp sesbania and sicklepod plants were thinned to 6 - 8 per pot and were grown to the cotyledonary-first leaf stage when used in the fungal spray application studies. All young seedlings were supplemented with a controlled-release (14:14:14, NPK) fertilizer (Grace Sierra Horticultural Products, Milpitas, CA, USA).

2.4. Spray Application of Mycelial Products

The fungal mycelial concentrations were prepared by blending (Virtis-45 blender, Gardiner, NY) the mycelial product that was stored refrigerated (4°C) after removal from shake flasks as described above, for 60 sec at 4000 rpm and 2°C - 4°C. This blended material yielded the 1.0 \times concentration. In tests spray applications to hemp sesbania and sicklepod seedlings, a 0.5 \times concentration of both MV and MV-Sector BSH mycelia was used. Dilutions were made with deionized H₂O, and Silwet L-77 was incorporated into the formulations to achieve a final surfactant concentration of 0.1% (v:v). Other treatments were: deionized H₂O, and Silwet at 0.10% in H₂O. After inoculation, the plants were placed in a high humidity chamber (28°C, 100% RH) for 15 h, and then transferred to greenhouse benches under conditions described above. The experiments were arranged in randomized complete block designs, containing three replications (5 pots each) and were repeated in time. Plants were excised at the soil line 72 h after treatment, and their fresh weights determined.

2.5. Seed Tests

To compare the efficacy of MV and MV-sector BSH on early seed growth, seeds of sicklepod and hemp sesbania were exposed to the shake culture products. Treatments were: control (H₂O); Silwet L-77 (0.10%, v:v); 1.0 \times MV; 0.25 \times MV; 1.0 \times MV-Sector BSH; and 0.25 \times MV-Sector BSH. Un-germinated seeds (5 of each weed species) were allowed to imbibe aliquots of each treatment solution for 12 h in the dark at 22°C - 24°C. The seeds were then removed and placed on germination paper discs (moistened

with H₂O) contained in Petri dishes, and growth (shoot + root length) of the imbibed seeds was determined after an additional 65 h incubation under dark conditions at the above temperature. These tests were performed in triplicate and repeated in time.

2.6. Leaf Disc Bioassay for Efficacy Determination

Discs were cut from greenhouse-grown kudzu and Palmer amaranth plant leaves using a cork borer (14 mm) and placed (one disc per well) in 24-well microtiter plates. Treatments consisted of adding 50 µL of either, deionized H₂O, Silwet (0.10%), or solutions of various concentrations of MV or MV-Sector BSH. The plates were incubated under continuous light (100 µmol·m⁻²·s⁻¹) in an environmental room at 26°C - 28°C for 90 h. The test was visually assessed for chlorosis/necrosis at various times after treatment, and at the end of the test (90 h). A modified visual disease rating scale [42] of 0 to 5 was utilized, where 0 = no injury and 5 = total chlorosis/necrosis (mortality). The tests were performed in triplicate and repeated in time.

2.7. Protein Extraction and SDS-PAGE

Equal aliquots of MV and MV-Sector BSH shake cultures were weighed and centrifuged at 15,000 ×g for 10 min. The mycelial pellet tissue (0.5 g fresh weight) was ground with 2.0 ml extraction buffer in a chilled glass homogenizer. Extraction buffer consisted of 62.5 mM Tris-HCl, pH 6.8 and 40% glycerol as modified from Bio-Rad Buffer Formulations Bulletin 6199, Rev A. After homogenation of the fungal mats, the samples were subjected to 4 alternating freeze: sonication cycles (10 min freezing at -80°C: 10 min sonication). After the last sonication cycle the homogenate was centrifuged at 15,000 ×g at 4°C for 15 min and the supernatant was retained for protein analysis. Since the protein concentration in culture supernatants of the organisms was too low for adequate detection on electrophoretic gels, an acetone precipitation protocol was used. Generally, 1332 µL of cold acetone (80% in ultrapure H₂O; -20°C) was added to 333 µL of each supernatant contained in acetone-compatible centrifuge tubes. The tubes were vortexed, followed by refrigeration (-20°C) for 60 min. The tubes were then centrifuged 10 min at 15,000 ×g at 4°C and the supernatant carefully decanted. The protein pellets were washed once in cold 80% acetone, vortexed and centrifuged 10 min at 15,000 ×g at 4°C. The supernatant was decanted and the pellets washed again with 50% acetone, followed by centrifugation. Then the supernatant was decanted and a fine tipped pipet used to remove any excess liquid, followed by air-drying in a fume hood for about 10 min. Then buffer (as above) was added and pellets dispersed and mixed with a pipet. Protein levels in samples were quantified (Thermo Fisher Pierce 660 nm Protein Assay). Following 1:1 dilution with 2× Laemmli Sample Buffer containing 2-mercaptoethanol aliquots, the samples were boiled 5 min and 25 µg total protein of each sample were fractionated by SDS polyacrylamide gel electrophoresis on a 4% - 20%, TGX Stain-Free Criterion gel (BioRad Corp., Van Nuys, CA). The gel was run for 45 min at constant voltage (200 V). Gels were imaged on a ChemiDoc MP imager using the stain-free protein gel protocol on Image Lab Software. Molecular weight marker

proteins (Precision Plus, Unstained) were electrophoresed in separate lanes as size standards (Bio-Rad Corp., Van Nuys, CA). Samples were analyzed in duplicate and four separate gels were electrophoresed.

2.8. Statistics

Values presented are means of replicated experiments, repeated in time. When significant differences were detected by the *F*-test, means were separated with Fisher's protected LSD (FLSD) test at the 0.05 level of probability. All data were analyzed using SAS (Version 9.1, SAS Institute, Inc., Cary, NC, USA).

3. Results and Discussion

Growth and Isolation of Sector

When MV is grown on PDA under alternating 12 h light/12 h dark cycles, concentric rings [spore (dark pigmentation) and mycelial (light) areas] are produced (**Figure 1(a)**). MV-Sector BSH formed during routine maintenance culture of MV (**Figure 1(a)**) and the sector was isolated and grown on PDA under the alternating light/dark regime as above (**Figure 1(b)**). No rings or dark pigmented areas corresponding to spore production were observed during growth for several days. Microscopic examination of the white biomass of the sector confirmed that only mycelial growth occurred and that no spores were formed. Over several months of subculture, the sector remained genetically stable, retained high virulence and exhibited no genetic reversion to the wild-type parent (data not shown). Growth of MV and MV-Sector BSH were very similar when grown on PDA or in shake-culture on soy flour: cornmeal: sucrose as described in the Material and Methods. This is the first report of a non-spore-producing mutant derived from this bioherbicidal strain of MV.

Comparative Efficacy Studies of MV and MV-Sector BSH

Spray application of MV and MV-Sector BSH mycelial preparations on intact hemp sesbania and sicklepod seedlings generally demonstrated equal visual bioherbicidal activity on both species, 72 h after treatment (**Figure 2**). Injury symptoms were growth reduction, chlorosis, necrosis and desiccation. Fresh weight analysis of seedling shoots (excised at soil line) treated with H₂O and with Silwet L-77 (0.10%; v:v) were not significantly different from each other in either species and plant growth in the MV and MV-Sector BSH treatments were reduced by 20 and 15%, in hemp sesbania and sicklepod, respectively (**Figure 3**).

Previous studies have examined the effects of MV spore preparations on growth and development of hemp sesbania and sicklepod seeds [12], and were also chosen for studies here. The effects of imbibing weed seeds (hemp sesbania and sicklepod) in mycelial preparations of MV and MV-Sector BSH showed that MV was slightly more toxic to hemp sesbania than to sicklepod and was also slightly more potent than the MV-Sector BSH to the early growth of both species (**Figure 4**).

Because Palmer amaranth and kudzu were subjects of previous MV studies [6] [16], they were also examined for effects of MV-Sector BSH. Application of the 1.0× concen-

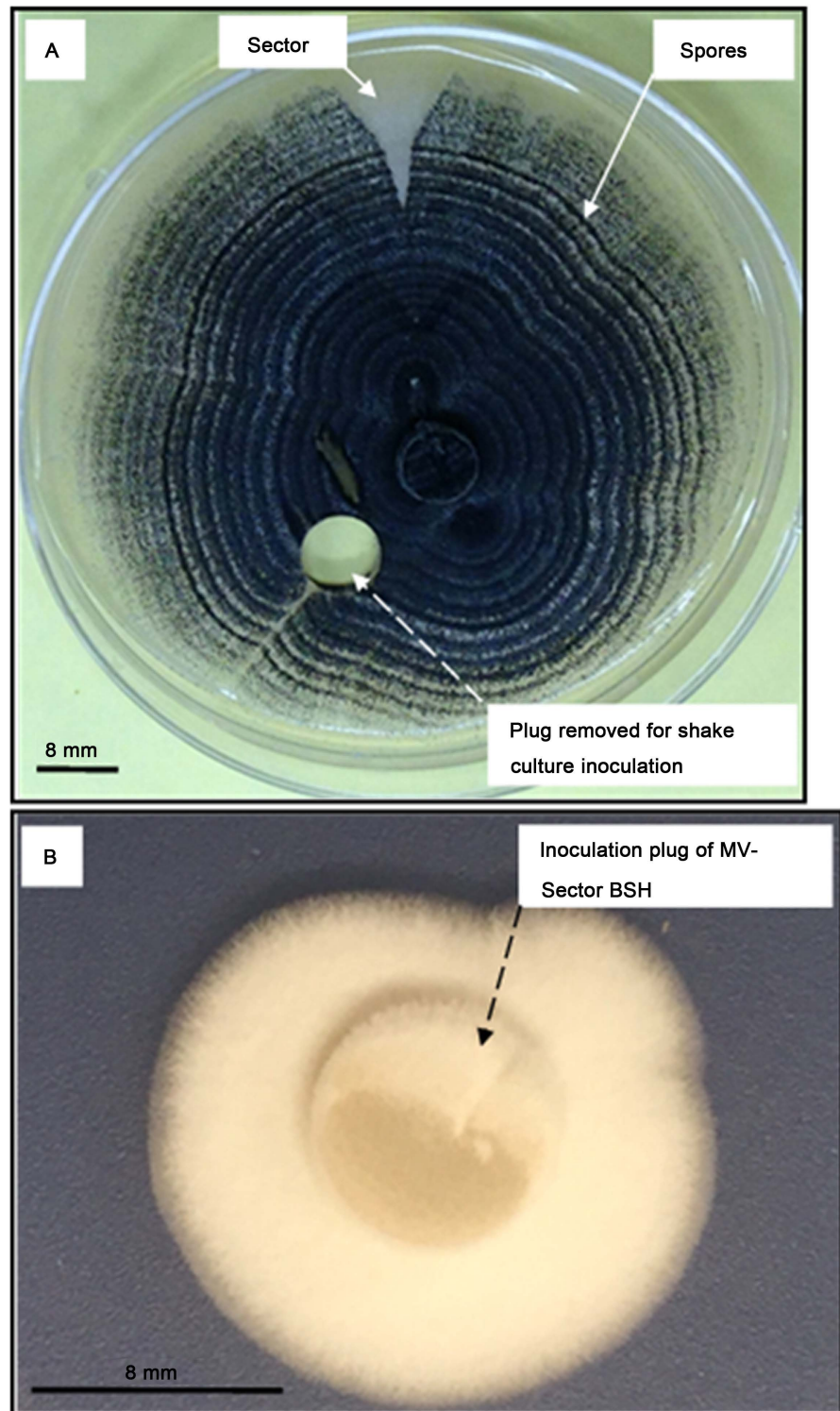


Figure 1. Development, isolation and growth of MV and MV-Sector BSH on PDA. (A) concentric growth rings of MV, depicting dark rings of spores, and non-sporulating area at outer edge of colony; also the development and growth of the sector (V-shape); circular cutout = plug removed for liquid shake culture inoculation; (B) growth of MV-Sector BSH on PDA; note whitish appearance, apparently due to absence of pigmentation associated with spore production.

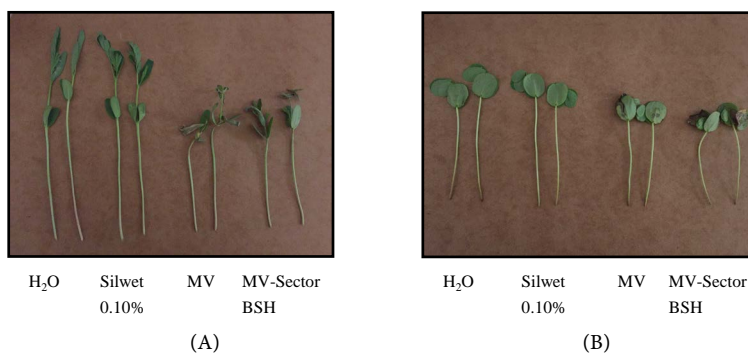


Figure 2. Photographs depicting effects of MV and MV-Sector BSH (each at 0.5× concentration) on sicklepod and hemp sesbania seedlings, 72 h after treatment. All treatments except H₂O contained Silwet surfactant (0.10%, v:v). (A) Hemp sesbania; (B) Sicklepod.

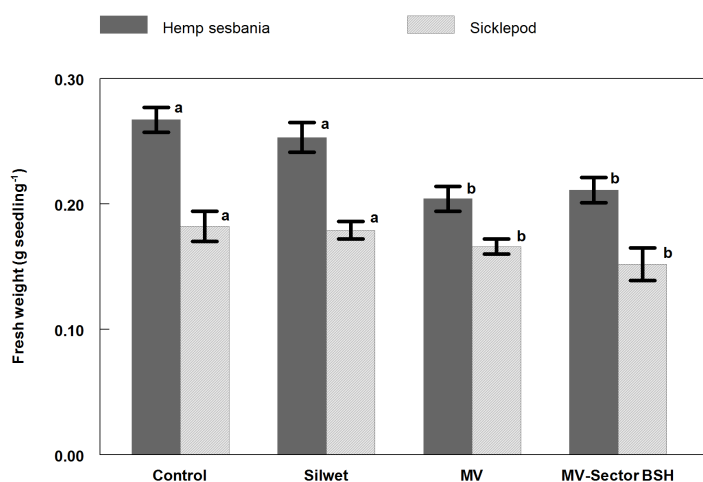


Figure 3. Effects on fresh weight accumulation of MV and MV-Sector BSH mycelial formulations (each at 0.5× concentration) applied to intact sicklepod and hemp sesbania seedlings, 72 h after application. Error bars represent 1 SEM. Different letters on histogram bars within species denote statistical significance at $P = 0.05$ (based on FLSD).

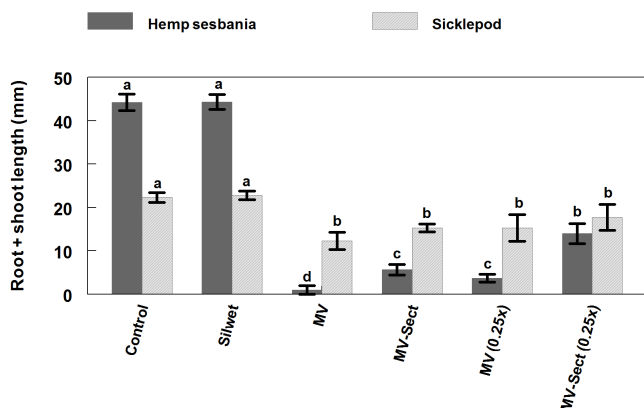


Figure 4. Growth effects of MV and MV-Sector BSH mycelial formulations applied to seeds of sicklepod and hemp sesbania, 72 h after imbibition. Error bars represent 1 SEM. Different letters on histogram bars within species denote statistical significance at $P = 0.05$ (based on FLSD).

tration of mycelial preparations of MV and MV-Sector BSH to leaf discs of glyphosate-resistant Palmer amaranth and kudzu plants showed that they generally caused equivalent injury on these species (Table 1). However, the sector was slightly less virulent than MV on Palmer amaranth at both concentrations. Application of lower concentrations of the bioherbicides (0.25×) resulted in less injury and the injury caused by the sector was slightly lower than MV in both species.

Protein Profile Studies

Comparison of SDS-PAGE protein profiles on gels of MV and the sector cell extracts, demonstrated that many bands were similar with respect to presence and abundance (Figure 5(a) and Figure 5(b)). However, some differences were observed and generally more bands appeared to be in greater relative abundance in the MV-Sector BSH versus the MV parent gel (Figure 5(a) and Figure 5(b)). Protein bands 1 and 3 were more abundant in MV (Lane a) versus MV-Sector BSH (Lane b), whereas four bands (2, 4, 5 & 6) were more abundant in the sector compared to its parent. Comparison of the protein profiles of the liquid culture supernatants of un-inoculated media (Lane e) with supernatants of the MV and MV-Sector BSH (Lanes c & d, respectively) showed that both organisms were able to transform media proteins. Some differences in protein bands in the media culture supernatants of MV and MV-Sector BSH were observed, possibly indicating differential substrate preference of the organisms for proteins supplied by the media, and/or to variance in the production of extracellular proteins (enzymes). Other strains of *Myrothecium verrucaria* are known to produce various hydrolytic enzymes including xylanases [43] [44], pectinases [44] [45], and proteases [44] [46]. Such enzymes play important roles in determining host range and virulence. The nature of these protein differences will need to be examined using native gels and 2-D gel electrophoresis to determine any possible role(s) as virulence factors.

Early bioherbicidal studies on spore formulations of this parent strain of MV showed that the fungus produced trichothecenes that have mammalian toxicity [1] [7] [29]. Another *M. verrucaria* isolate from leafy spurge (*Euphorbia esula* L.) exhibited a dissimilar weed host specificity range, but also produced trichothecenes [47] [48]. Although

Table 1. Effects of application of MV and MV-sector mycelial formulations on leaf discs of glyphosate-resistant Palmer amaranth and kudzu plants, 72 h after application.

Treatment	Palmer amaranth	Kudzu
Control	0.00 (0.0) a	0.00 (0.00) a
Silwet	0.00 (0.0) a	0.50 (0.04) b
MV	3.45 (0.04) b	3.95 (0.04) c
MV-(0.25×)	2.50 (0.0) d	3.25 (0.22) d
MV-Sector BSH	3.15 (0.11) c	4.00 (0.00) c
MV-Sector BSH (0.25×)	2.00 (0.0) e	2.65 (0.18) e

Visual evaluation of injury/infectivity of MV and MV-Sector BSH. A modified Horsfall [42] rating scale was used as described in Materials and Methods. Data presented are means followed by standard error of means in parenthesis. Values followed by the same letter are not significantly different at $P = 0.05$ (FLSD).

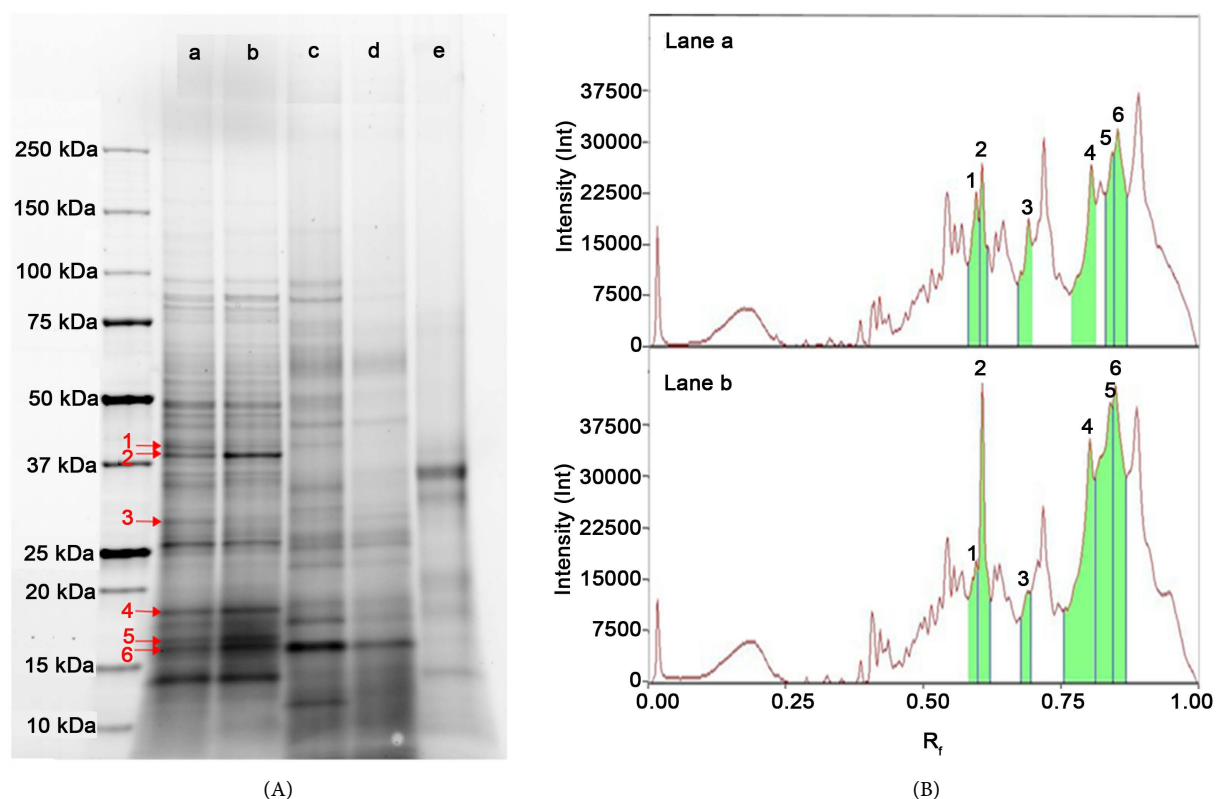


Figure 5. SDS-PAGE of protein extracts of MV and MV-Sector BSH. Gel and electrophoresis protocol is described in Materials and Methods. (A) = electrophoresed samples on gels, including molecular weight standards; Lane a = MV; Lane b = MV-Sector BSH; Lane c = MV culture supernatant; Lane d = MV-Sector BSH culture supernatant; Lane e = un-inoculated culture supernatant. Red arrows (A) indicate selected protein bands with significant differences between MV (lane a) and MV-Sector BSH (Lane b). (B) = lane intensity profiles (normalized with respect to Lane a) as analyzed with Image Lab Software, Version 4.1 (Bio-Rad, Hercules, CA).

MV possessed desirable bioherbicidal traits (high virulence and broad weed host range), production of undesirable mycotoxins remained problematic with regard to the development of a commercial product. Approaches to possibly reduce or eliminate these mycotoxins were considered [49], and cultural manipulation of carbon and nitrogen source/level [50], spore clean-up or washing [51] and growth of MV in liquid culture without spore production [40] were found successful. Of these techniques, the mycelial form of MV (produced in liquid shake-flask or in fermenters) was found to be the most efficient for further studies [40].

We have also previously shown that MV has produced other mutant sectors that had varying degrees of virulence when tested on kudzu [14]. In those tests a white sector was found, but it exhibited no bioherbicidal efficacy on kudzu and was not tested on other weeds. In these present studies a white mutant (MV-Sector BSH) was discovered and isolated, and shown to be virulent in several tests on hemp sesbania, sicklepod, kudzu and glyphosate-resistant Palmer amaranth. The fact that this virulent sector is stable, does not produce spores and exhibits bioherbicidal activity on major weeds indicates it may be a useful stand-alone bioherbicide or as a tool for future comparative

studies with MV. Since this sector reproduces only through mycelial growth, it is assumed that the production of trichothecenes is also mitigated. However, this will have to be monitored. Future studies can be envisioned, including more extensive host range tests with weeds (and crops), interactions with herbicides and tests for efficacy in the field. Since some proteins differed in MV-Sector BSH cells and in culture supernatants compared to those of MV, their identity and possible function in infection and virulence processes should be examined.

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