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ORIGINAL PAPER

Bioherbicidal activity from washed spores of *Myrothecium verrucaria*

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Abstract The fungal plant pathogen, *Myrothecium verrucaria,* is highly virulent to several important weed species and has potential utility as a bioherbicide. However the production of macrocyclic trichothecene mycotoxins by this fungus presents significant safety concerns. It was discovered that trichothecenes are removed from *M. verrucaria* spores by repeated washes with water. These washed spores retained bioherbicidal efficacy against kudzu when tested in field trials and on sicklepod when tested under greenhouse conditions. Changes in the growth medium combined with washing spores with water resulted in greater than 95% reduction in roridin A and verrucarin A. Washing spores reduced trichothecene concentrations in spore preparations with no significant effect on plant biomass reduction, thus demonstrating the possibility of *M. verrucaria* formulations with improved safety to researchers, producers and applicators.

Keywords Bioherbicide · Sicklepod · *Cassia obtusifolia* · kudzu · *Pueraria lobata* · Roridin · Verrucarin

Introduction

A strain of the fungal plant pathogen, *Myrothecium verucarria,* was isolated from sicklepod (*Cassia obtusifolia*) and shown to be particularly virulent against several weedy plant species and potentially useful as a bioherbicide (Walker and Tilley 1997). Subsequent experiments demonstrated efficacy against several morning glory species (*Ipomea spp.*) (Hoagland et al. 2011), leafy spurge (*Euphorbia esula*) (Yang and Jong 1995), redvine (*Brunnichia ovata*) and trumpetcreeper (*Campsis radicans*) (Boyette et al. 2006), and kudzu (*Pueraria lobata*) (Boyette et al. 2002). The greatest impediment to the commercial development of this fungal bioherbicide is the production of macrocyclic trichothecene mycotoxins (Hoagland et al. 2007; Anderson and Hallett 2004). Various strains of *M. verrucaria* have been reported to produce an assemblage of trichothecenes, including roridin E and H, and verrucarin A, B, and J (Abbas et al. 2001; Millhollon et al. 2003). Since these mammalian toxins are also phytotoxic (Cutler and Jarvis Jarvis et al. 1985; Abbas et al. 2002), it is possible that they are the principle source of the observed bioherbicidal activity.

We previously documented that accumulation of macrocyclic trichothecene is suppressed in strain IMI 361390 by manipulation of the nutrient composition in solid media (Weaver et al. 2009b) or by growth in liquid fermentation (Boyette et al. 2008). In the present study we demonstrate that: (1) macrocyclic trichothecenes are removed from spores through washing with water; (2) washed spore preparations are bioherbicidal; and (3) there is significant phytotoxic activity from non-trichothecene metabolites.

Materials and methods

Spore production

M. verucarria (IMI 361390) was grown on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) or Vogel's-glucose (VMG) medium (Vogel, 1956; Weaver et al. 2009 b) at 28°C with a 12 h light/dark cycle in 28 × 21 cm autoclavable boxes with translucent lids (Market Labs,

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Caledonia, MI, USA). Spores were harvested after 7 days of incubation by scraping the surface with a sterile spatula while keeping the culture moistened under a layer of water. The spores and detached mycelia were briefly homogenized in a Waring blender and passed through a Miracloth filter (Calbiochem-Novabiochem San Diego,CA, USA) (Weaver et al. 2009b). For treatments requiring washed spores, the suspension was centrifuged at 2,500×*g* for 5 min. The supernatant was set aside and the spores were resuspended in sterile water and centrifuged again for a total of 3 washings.

Trichothecene analysis

Trichothecenes were extracted from spore preparations or filtrates by mixing with an equal volume of ethanol for 2 h and centrifuging at 14,000×*g* to remove particulates before analysis via HPLC (Weaver et al. 2009b). Samples were separated over a Kinetex PFP column (Phenomenex, Torrance, CA) on an Ultimate 3000 HPLC system with UV–VIS detection (Weaver et al. 2009b) and compared with commercially available standards (Sigma Chemical Company, St. Louis, MO).

Bioherbicidal activity

Field experiments were conducted on Dundee very fine sandy loam (Aeric Ochraqualf) soil June 27, 2005 and August 5, 2005 at the USDA-ARS, Southern Weed Science Experimental Farm, Stoneville, Mississippi, USA. Plots consisted of single rows (3 × 0.5 m) planted with scarified kudzu seed (Adams-Brisco Seed Co., Jackson, GA 30233) at 100 seeds m^{-1} of row. Treatments consisted of: (1) washed *M. verrucaria* conidia at 2.0×10^7 conidia ml^{-1} in distilled water or with 0.20% (v/v) Silwet L-77 surfactant; (2) unwashed conidia at 2.0×10^7 conidia ml^{-1} in distilled water or with 0.20% Silwet L-77, (3) 0.20% Silwet L-77 only, (4) glyphosate herbicide (1.12 kg ha^{-1}, Roundup Ultra^{TM}), and (5) distilled water only. The plants, approximately 12 cm in height, were sprayed until fully wetted (approximately 450 l ha^{-1}), resulting in inoculum densities of 9.0×10^{12} conidia ha^{-1} in fungal treated plots. Applications were made at midday with a handheld pressurized sprayer. For the June 27 experiment, environmental conditions at the time of inoculation and for 24 h following treatment were: temperature at inoculation, 33°C with a RH of 56%. The high temperature for the 24 h period was 34°C, and the low temperature was 23°C. Maximum RH was 94%, with light dew the following night that lasted about 3 h. For the August 5 experiment, environmental conditions at the time of inoculation and for 24 h following treatment were: temperature at inoculation, 34°C with a RH of 39%. The high temperature for the 24 h period was 36°C, and the low temperature was 24°C. Maximum relative humidity was 94%, with light dew that lasted about 5 h. A randomized complete block design was utilized, and the treatments were replicated three times. Mortality data were collected 7 days after treatment from 0.5 m^2 subplots. Mortality percentages were determined by counting the number of living kudzu plants prior to inoculation divided by the number of living kudzu plants after inoculation. Data from the June and August experiments were pooled following subjection to Bartlett's test for homogeneity, and analyzed using analysis of variance. Percentage data of kudzu control were subjected to arcsin, square root transformation prior to analysis. Significant differences were determined using Fisher's Protected Least Significant Difference (FLSD) at alpha = 0.05 (Steele et al. 1997). The fungus was isolated from diseased kudzu leaf and stem tissues by surface sterilizing excised sections in 0.05% NaOCl for 1 m, then placing sections on potato-dextrose agar (PDA) amended with chloramphenicol (0.75 mg ml^{-1}) and streptomycin sulfate (1.25 mg ml^{-1}).

Seeds of sicklepod (*Cassia obtusifolia*) were planted in Metro Mix 360 potting mix (Sun Gro Horticulture, Bellevue, WA, USA) and grown to the stage of 2 fully expanded compound leaves. Suspensions of *M. verrucaria* spores were prepared at concentrations of 2×10^7 or 4×10^6 spores ml^{-1} or supernatant from washed spores was mixed with Induce non-ionic spray adjuvant (Helena Chemical Company, Collierville, TN, USA) at a concentration of 0.25% surfactant (v/v). Individual plants were sprayed with these mixtures with a handheld trigger-action sprayer until run-off and transferred to a dew chamber overnight before returning to the greenhouse. After 5 days the plants were visually rated on a disease severity scale (1 = dead plants; 2 = no living leaves, some green stems; 3 = lesions or limited necrosis; 4 = healthy plants). After 7 days seedlings were cut at the soil line and air dried to measure dry plant weight. After a square root, arcsin transformation, the bioherbicidal activity was analyzed via the proc mixed function (SAS v. 9.1, Cary, NC, USA) and the pdmix800 macro (Saxton, 1998), which generated means, standard deviations and letter groupings of protected pairwise comparisons for each response variable (biomass or visual disease rating). Results were back-transformed to the original measurements for presentation.

Results and discussion

In field experiments, kudzu plants treated with either the washed or unwashed *M. verrucaria*/surfactant mixtures developed severe leaf and stem necrosis within 24 h following inoculation with control levels of 75 and 78%, respectively. Disease severity increased with time causing

95 and 98% mortality after 96 h in plants treated with washed and unwashed *M. verrucaria*, respectively, and 100% mortality of plants in both treatments after 120 h (Fig. 1 and Table 1). These rates of control were similar to that achieved with glyphosate (95%) (Table 1). Only 15% mortality occurred to plants treated with washed or unwashed *M. verrucaria* spores in water only (Table 1), consistent with other reports that a surfactant is required for expression of this bioherbicidal activity (Hoagland et al. 2007; Weaver et al. 2009a). No regrowth was observed on kudzu plants treated with the *M. verrucaria*/Silwet-L-77

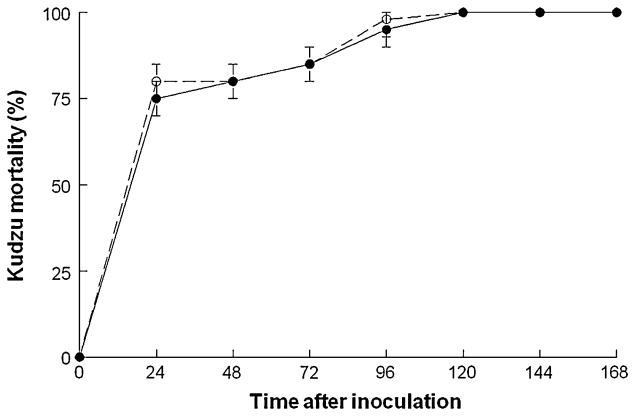

Fig. 1 Biological control of kudzu in a field bioassay. *Myrothecium verrucaria* conidia were applied at a concentration of 2.0×10^7 conidia ml^{-1} in 0.02% Silwet L-77 surfactant. *Solid spheres with solid lines* represent washed MV conidia; *open spheres with dashed lines* represent unwashed MV conidia. *Error bars* represent LSD values at $P = 0.05$

Table 1 Bioherbicidal activity of *M. verrucaria* spore preparations on kudzu

Treatment[a]	Kudzu mortality (%)[b]
Washed MV + water[c]	15
Unwashed MV + water[c]	15
Washed MV + water[c] + surfactant[d]	100
Unwashed MV + water[c] + surfactant[d]	100
Glyphosate[e]	95
Surfactant + water	0
Untreated (water only)	0
$FLSD_{05} = 9$	

[a] Applications were made administered at a rate of 450 l ha^{-1} with a hand-held pressurized sprayer

[b] Mortality measured 7 days after treatment. Plants were considered 'killed' when 100% necrosis occurred to individual plants

[c] 2.0×10^7 conidia ml^{-1}

[d] 0.2% Silwet L-77 surfactant

[e] Roundup Ultra™ herbicide at 1.12 kg acid equivalent glyphosate ha^{-1}

suspensions during the experiments. The fungus sporulated visibly on infected tissue. The fungus re-isolated from these inoculated plants killed greenhouse-grown kudzu seedling when inoculated onto them, thus fulfilling Koch's postulates. No disease or mortality occurred on untreated control plants, or to plants treated with surfactant only (Table 1).

To more directly test the bioherbicidal activity of *M. verrucaria* and the role of trichothecenes in weed control, we treated seedlings of sicklepod with spores at two concentrations, using spores from two different media that support different levels of trichothecene production, and applied the inoculum as washed or as unwashed spores. The water used to wash the spores was also used as inoculum. The trichothecene levels in these preparations and the associated bioherbicidal activity is presented in Table 2 and a chromatograph indicating the relative amounts of trichothecenes in each spore preparation is presented (Fig 2.).

There are other, unnamed peaks with absorbances at 260 nm, which is characteristic of macrocyclic trichothecenes (Fig. 2). Some of the other peaks have been tentatively identified as roridin D, E and H, and verrucarin J (Weaver et al. 2009b), which were also similarly reduced by washing or by changing the growth medium from PDA to VMG (data not shown). Macrocyclic trichothecenes are a large family of structurally related mycotoxins. The toxicological properties of members of this family are not all well documented, but are cause for concern (Abbas et al. 2002).

Complete necrosis and greater than 90% reduction in biomass was obtained with several of the sicklepod treatments. Depending on the metric (disease rating or shoot dry weight), the highest level of control was obtained with washed or with unwashed spores, at high or low concentrations, and with spores produced on PDA or VMG. There was no statistical separation between many of the treatments for disease ratings or for shoot dry weight reduction. Washing spores with water reduced the concentration of roridin A and verrucarin A by three- to six-fold, and caused a small reduction in efficacy, based on visual disease ratings, and a statistically non-significant effect on plant weight (Table 2 and 3). Table 3 outlines the magnitude various treatment variables had on the two response variables (disease rating and shoot biomass) and the level of statistical significance of each variable. Trichothecene concentration was at least seven-fold lower in spore suspensions from VMG medium than from PDA medium, with no significant change in visual disease ratings, but PDA- grown spores caused a greater reduction in plant biomass (Table 2, 3). Overall, washed spores from VMG had less than 5% as much roridin A and verrucarin A as unwashed spores from PDA while still demonstrating substantial bioherbicidal activity (Table 2).

This observed bioherbicidal activity in the field trial with kudzu and in the greenhouse experiment with

Table 2 Bioherbicidal activity of *M. verrucaria* spore preparations on sicklepod

Growth medium	Concentration of spores	Washed or unwashed	Visual disease rating[a]	Individual plant dry weight (g)[b]	Roridin A (ppm)	Verrucarin A (ppm)
PDA	2×10^7	Unwashed	1.2 D	0.03 C	18	86
PDA	2×10^7	Washed	2.1 BC	0.02 C	5	28
PDA	4×10^6	Unwashed	1.4 CD	0.01 C	nd[c]	nd
PDA	4×10^6	Washed	2.5 B	0.08 BC	nd	nd
PDA	0 (filtrate)[d]		2.5 B	0.04 C	10	50
VMG	2×10^7	Unwashed	1.1 D	0.04 C	5	15
VMG	2×10^7	Washed	2.5 B	0.04 C	1	5
VMG	4×10^6	Unwashed	2.5 B	0.11 B	nd	nd
VMG	4×10^6	Washed	2.5 B	0.12 B	nd	nd
VMG	0 (filtrate)		2.5 B	0.06 BC	4	14
Surfactant-only control	0		4 A	0.36 A	nd	nd
			Pairwise comparisons			
PDA			1.7 A	0.032 A		
VMG			2.0 A	0.065 B		
	High		0.5 A	0.03 A		
	Low		0.7 B	0.08 B		
		Unwashed	1.4 A	0.05 A		
		Washed	2.4 B	0.06 A		

[a] Disease rating on 4 point scale from 1 = dead plants to 4 = no apparent injury or disease. Values followed by the same letter are not significantly different by least significant difference test (a = 0.05)

[b] Shoot dry weights 7 days after treatment. Values followed by the same letter are not significantly different by least significant difference test (a = 0.05)

[c] not determined

[d] Supernatant resulting from pelleting spores by centrifugation in the washing process

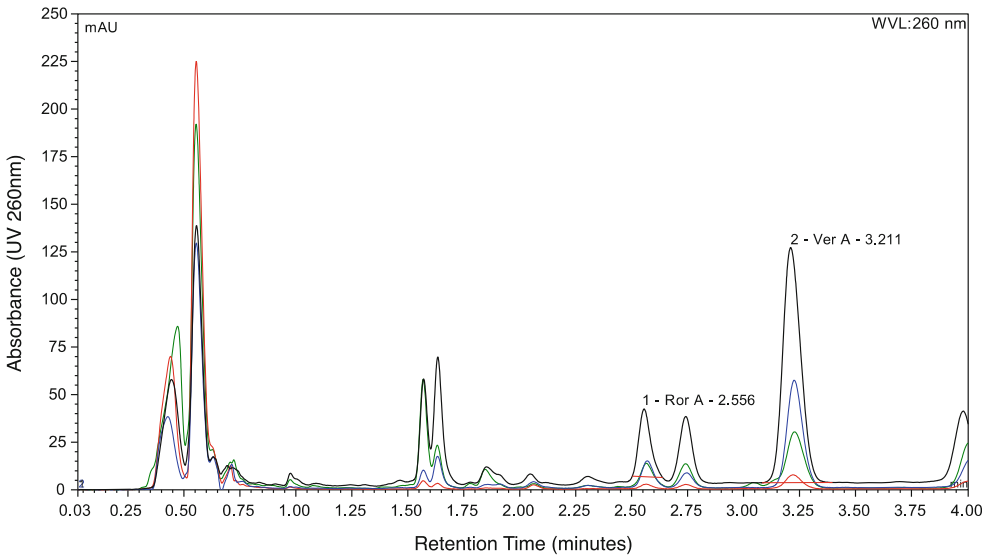

Fig. 2 Detection of trichothecenes by HPLC. Chromatograph of ethanol extracts of spores grown on PDA (*black line*), washed spores from PDA (*blue line*), grown on VMG (*green line*) or washed spores from VMG (*red line*)

sicklepod is in contrast to previously reported efficacy (Anderson and Hallett, 2004), where washed spores had very limited activity on some tested weed species, including sicklepod. The authors of that study concluded that the *M. verrucaria* efficacy "is primarily caused by the activity of metabolites produced by the fungus in culture" and carefully quantified the activity of the metabolites in crude extracts, although phytotoxic metabolites were not

Table 3 Analysis of significant effects on bioherbicidal activity of *M. verrucaria* spore preparations on sicklepod

Effect	Visual disease rating		Biomass reduction	
	F value	Pr > F	*F* value	Pr > F
Growth medium	1.1		6.1	[a]
Inoculum concentration	4.5	[a]	9.5	[a]
Washing spores	10.2	[a]	1.1	
Medium a wash interaction	0.1		0.6	

[a] Pr > F is <0.05

identified. Similarly, Millhollon et al. (2003) reported bioherbicidal activity from a strain of *M. verrucaria* that survived autoclave treatment. As a part of that study, mycotoxins were quantified and while several trichothecenes were noted at high concentrations in culture, only roridin A and verrucarin A were found in conidia, albeit at much lower concentrations and undetectable 48 h after application. Abbas et al. (2001) also reported bioherbicidal activity of *M. verrucaria* against kudzu, but failed to detect trichothecenes in situ. More recently, we have demonstrated an ELISA method to quantify trichothecenes in plant tissues and have observed translocation when trichothecenes were applied to plants (Hoagland et al. 2008).

A central question to the further development of *M. verrucaria* is the essential nature of trichothecene mycotoxins to the bioherbicidal activity. It is established that some trichothecenes are phytotoxic (Abbas et al. 2002), and reports of symptom development are suggestive of the action of one or more phytotoxins (Walker and Tiley 1997; Jarvis et al. 1985). Millhollon et al. (2003) noted the production of trichothecenes by another strain of *M. verrucaria* and that autoclaved conidia were significantly phytotoxic to morningglory, although not as efficacious as live conidia. Similarly, Anderson and Hallett (2004) noted greater bioherbicidal activity from a crude or a cell-free extract than from washed spores and concluded that the primary cause of the bioherbicidal efficacy was through the action of phytotoxic metabolites. This is not proof, however, that trichothecenes are the sole phytotoxin produced by *M. verrucaria*, or that trichothecenes are essential for bioherbicide activity.

Millhollon et al. (2003) detected only traces of trichothecenes 24 h after application, and none after 48 or 96 h. Similarly, Abbas et al. (2001) were unable to detect trichothecenes after application to kudzu. Thus, the trichothecene-related safety concerns are concentrated on the production and application of *M. verrucaria* (Anderson and Hallett, 2004), and it was proposed that, in the interest of safety, cultural conditions should be identified that mitigate trichothecene production, although the feasibility of this approach was considered "unlikely." Since then, we have reported bioherbicidal activity of *M. verrucaria* formulations in the absence of detectable trichothecenes when the fungus was grown in liquid culture (Boyette et al. 2008b) and that trichothecene levels could be greatly reduced by altering the nutrient composition of agar-based solid substrate (Weaver et al. 2009b). In the present study we have demonstrated that significant levels of trichothecene mycotoxins can be removed by washing spores with water. While some phytotoxic compound(s) are removed in the process, the washed spores retain bioherbicidal efficacy. When the washed spores are obtained from VMG medium, more than a 95% reduction in measured mycotoxins was achieved relative to the unwashed spores from PDA. Use of appropriate cultural conditions and washing of spores can thus mitigate the hazards associated with this bioherbicide.

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