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Macrocyclic trichothecenes are undetectable in kudzu (*Pueraria montana*) plants treated with a high-producing isolate of *Myrothecium verrucaria*

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Abstract

Myrothecium verrucaria was found to be an effective pathogen against kudzu grown in the greenhouse and the field. *M. verrucaria* produced large amounts of macrocyclic trichothecenes when cultured on solid rice medium, including epiroridin E (16.8 mg/g crude extract), epiisororidin E (1 mg/g), roridin E (8.7 mg/g), roridin H (31.3 mg/g), trichoverrin A (0.6 mg/g), trichoverrin B (0.1 mg/g), verrucarin A (37.4 mg/g), and verrucarin J (2.2 mg/g). Most of these toxins were also isolated from *M. verrucaria* spores and mycelia grown on potato dextrose agar medium, including epiroridin E (32.3 mg/g), epiisororidin E (28.6 mg/g), roridin E (0 mg/g), roridin H (60 mg/g), trichoverrin A (1.3 mg/g), trichoverrin B (1.8 mg/g), verrucarin A (13.8 mg/g), and verrucarin J (131 mg/g). When *M. verrucaria* was cultured on liquid media, the numbers but not the amounts of toxins decreased. Only epiroridin E (28.3 mg/g), epiisororidin E (29.6 mg/g), verrucarin B (195 mg/g) and verrucarin J (52.6 mg/g) were measured when the fungus was cultured on cornsteep medium. On soyflour–cornmeal broth *M. verrucaria* produced several toxins, including epiroridin E (58.1 mg/g), epiisororidin E (5.8 mg/g), verrucarin B (29.9 mg/g) and verrucarin J (32 mg/g). In contrast, no macrocyclic trichothecenes were detected by HPLC analysis of plant tissues of kudzu, sicklepod, and soybean treated with aqueous suspensions of *M. verrucaria* spores formulated with a surfactant. Chloroform–methanol extracts of kudzu leaves and stems treated with *M. verrucaria* spores were less cytotoxic to four cultured mammalian cell lines than the corresponding extracts from control plants. Purified macrocyclic trichothecenes (verrucarin A and T-2 toxin) were very cytotoxic to the same cell lines ($\leqslant 2$ ng/ml). These results show that neither intact macrocyclic trichothecenes nor toxic metabolites could be detected in plant tissues after treatment with *M. verrucaria* spores. These results argue for both safety and efficacy for the use of *M. verrucaria* in biological control of kudzu and other noxious weeds, and support proceeding to animal feeding trials for further evaluation of safety.

Keywords: Macrocyclic trichothecenes; Mycotoxins; Natural products; Kudzu (*Pueraria montana*); Mycoherbicide; *Myrothecium verrucaria*; Phytotoxins; Phytotoxicity; Cytotoxicity; Biological control

1. Introduction

Myrothecium verrucaria is a fungal pathogen that has been known to attack important crop and ornamental plants including beet, marigold, sunflower, zinnia, peanut, cotton, red clover, and potato (Cunfer et al., 1969; Walker and Tilley, 1997). *M. verrucaria* also attacks important weed plants such as redroot pigweed, cocklebur, velvetleaf, and johnsongrass (Yang and Jong, 1995a,b; Walker and Tilley, 1997). A few reports have shown the potential of various isolates of *M. verrucaria* for use in biological control of weeds (Sutton and Peng, 1993; Zhang et al., 1994). Recently, it has been reported (Boyette et al., 1999) that *M. verrucaria* is also pathogenic to kudzu (*Pueraria montana*). Kudzu is an exotic invasive vine that is responsible for massive economic and aesthetic losses every year in the southern US. Kudzu, native to eastern Asia, was introduced into the eastern and southern US in the 1800s (Beckwith and Dangerfield, 1996; Piper, 1920). It was originally promoted for

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erosion control and as an inexpensive forage for livestock (Bailey, 1944; McKee and Stephens, 1943). It is now present from Florida to New York, westward to central Oklahoma and Texas, with heavy infestations in Alabama, Georgia and Mississippi (Everest et al., 1991; US Congress, 1993). Kudzu grows up the sides of buildings, along fences and on telephone poles, and dies in the winter, leaving behind ugly, brown foliage (Miller, 1997). *M. verrucaria* has been shown in preliminary experiments to cause 90% mortality to kudzu plants in the greenhouse and the field (Boyette et al., 1999). Other weed species such as sicklepod, leafy spurge (*Euphorbia esula* L.) (Yang and Jong, 1995a) and plumeless thistle (*Carduus acanthoides* L.) (Yang and Jong, 1995b) are also susceptible to isolates of *M. verrucaria*. Fortunately, the pathogen has shown no toxic or pathogenic effects to oak, sycamore and other forestry trees (unpublished data). The potential of *M. verrucaria* spore suspensions as a bioherbicide has been limited by its broad host range, and because isolates produce macrocyclic trichothecene toxins in culture (Jarvis et al., 1985; Jarvis, 1991; Grove, 1993). The toxins include verrucarin A, B and J, and roridin A, D, E, H and J, which are known to be toxic to cultured mammalian cells as well as phytotoxic (Mortimer et al., 1971; Busam and Habermehl, 1982; Cutler and Jarvis, 1985; Jarvis et al., 1988; Schiefer et al., 1989; Jarvis, 1991). However, macrocyclic trichothecenes have not been isolated from plants infected with high toxin-producing strains of *Myrothecium* sp. (Bean et al., 1984). The present study was designed to evaluate the production of macrocyclic trichothecenes by *M. verrucaria* on a variety of culture media, and to determine the fate of these toxins in kudzu plants treated with spore suspensions of the same isolate.

2. Results and discussion

A culture of the fungus *M. verrucaria,* originally obtained from naturally infected sicklepod (*Senna obtusifolia* L.), exhibited potential application as a biocontrol agent for use against sicklepod and kudzu. Published studies (Jarvis et al., 1982; Bloem et al., 1983; Lee and Mirocha, 1984) and previous experience in this laboratory (Abbas et al., 1988) indicate that isolates of *M. verrucaria* vary widely in terms of toxin production when cultured on various media [solid, liquids, potato dextrose agar (PDA)]. Some isolates produced large numbers and quantities of toxins on solid media such as rice, whereas others produced more in liquid media. The toxin-producing capabilities of the *M. verrucaria* isolate used in this study were initially characterized with respect to its ability to produce macrocyclic trichothecene toxins on both solid and liquid media. *M. verrucaria* produced large quantities of numerous toxins when cultured on solid rice medium (Table 1). These toxins were epiroridin E, epiisororidin E, roridin E, roridin H, trichoverrin A, trichoverrin B, verrucarin A, verrucarin B, and verrucarin J. Several toxins were present at high levels in spores and mycelia of *M. verrucaria* grown on PDA, including epiroridin E, epiisororidin E, roridin H, trichoverrin A, trichoverrin B, verrucarin A, and verrucarin J (Table 1). *M. verrucaria* also produced macrocyclic trichothecenes when cultured in two different liquid media. However, fewer toxins were produced in liquid media than on solid rice substrate, or were present in spores and mycelia. The fungus produced the following four toxins in cornsteep liquor and soyflour liquid media: epiroridin E, epiisororidin E, verrucarin B and verrucarin J (see Table 1). Verrucarin A was not produced in either liquid medium.

In greenhouse experiments, the isolate of *M. verrucaria* was active in controlling kudzu in the absence of dew, using conidia suspensions formulated in 0.2% Silwet L-77 surfactant. Potted kudzu plants at the 3- to 5-true leaf stage developed disease symptomatology of *M. verrucaria* infection, including necrotic flecking, within 6 h after treatment with conidia suspensions at temperatures 28–35 °C. Disease symptoms progressed from infected leaves to necrotic stem lesions within 48 h. Based on the disease symptoms, treated kudzu plants were harvested for toxin analysis. At 24 h, infection percentages were 50% in treated trays with symptoms including wilted leaves and petioles and necrotic lesions. The roots of the same plants showed no disease symptomatology. At 72 h, infection was present in 70% of treated kudzu plants, which exhibited more pronounced disease symptoms than at 24 h, including necrotic lesions on stems, but the roots remained healthy. At 120 h, 95% of treated kudzu plants were diseased. From 168 h to 14 days, 100% of treated kudzu plants were diseased, while the roots remained healthy. All control kudzu plants for each time period remained healthy, and showed minimal or no damage from 0.2% Silwet L-77. Plants treated in the field, including kudzu, sicklepod, and soybean also showed symptoms of disease at either high or low dose treatment levels. At 72 h, low dose level (1.0×10^6 conidia/ml) plots had an infection rate from 20 to 30%, and high dose level (2.0×10^7 conidia/ml) plots were 80 to 100% infected. At 5, 7, 10, and 14 days, diseased and non-diseased tissues were taken for toxin analysis. The roots of the plants species in the field were not evaluated for disease symptoms or toxin content.

None of the plant tissues studied (leaves and stems and greenhouse plant roots) of kudzu, soybean or sicklepod contained any macrocyclic trichothecenes detectable by HPLC analysis at any time after treatment, despite their presence in the *M. verrucaria* spore and mycelia preparations used for treatment. Similarly, all kinds of tissues of each plant species (kudzu, soybean, sicklepod) were negative by HPLC analysis for other toxins examined in this study (Fig. 1). To eliminate the

possibility that toxic derivatives or metabolites were present in the tissues of treated plants, but were not detected by HPLC because their HPLC elution times differed from available standards, we examined tissue extracts for in vitro toxicity to cultured mammalian cells. The results of these studies are presented in Tables 2–4. Extracts from kudzu foliage tissues (leaves and stems) treated with fungal *M. verrucaria* at 2×10^7 conidia/ml in 0.2% Silwet L-77 generally caused less cytotoxicity to the four mammalian cell lines tested (Table 2) than extracts prepared in the same manner from untreated control tissues. Also, sicklepod and soybean foliage treated with fungal *M. verrucaria* at 2×10^7 conidia/ml in 0.2% Silwet L-77 generally caused less cytotoxicity to four cell lines tested (Tables 3 and 4) in comparison to extracts prepared in the same manner from untreated control tissues. We speculate that low levels of cytotoxicity observed in control tissues results from phytoalexins produced from progenitors during sampling and extraction, and the generally lower levels of cytotoxicity in *M. verrucaria*-treated tissues reflects prior depletion of phytoalexin progenitors in response to exposure to *M. verrucaria*. Consistent with this, the most toxic extract from *M. verrucaria*-treated kudzu tissues was obtained after 14 days recovery time following treatment (Table 2). The purified toxins verrucarin A,

Table 1
Toxin production by *Myrothecium verrucarin* grown in culture on various substrates

Toxin	Mycotoxin level (mg per gram extract)[a]						
	Solid rice media	Liquid[b]		Spores and mycelia[c]	Plant tissues[d]		
		CSL	SF		Kudzu	Sicklepod	Soybean
Epiroridin E	17	28	58	32	<0.01	<0.01	<0.01
Epiisororidin E	1.0	29.6	5.8	29	<0.01	<0.01	<0.01
Isororidin E	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Roridin E	8.7	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Roridin H	31.3	<0.01	<0.01	60	<0.01	<0.01	<0.01
Trichoverrin A	0.6	<0.01	<0.01	1.3	<0.01	<0.01	<0.01
Trichoverrin B	0.1	<0.01	<0.01	1.8	<0.01	<0.01	<0.01
Verrucarin A	37.4	<0.01	<0.01	14	<0.01	<0.01	<0.01
Verrucarin B	<0.01	195	30	<0.01	<0.01	<0.01	<0.01
Verrucarin J	2.2	57	32	131	<0.01	<0.01	<0.01

[a] Limit of detection was 5 ng.
[b] CSL = cornsteep liquor; SF = soyflour–cornmeal broth.
[c] Spores (conidia) and mycelia were grown in plastic Petri dishes (9 cm inside diameter) containing Difco potato dextrose agar (PDA).
[d] Plant tissues include every part of the plant (leaves, stems, and roots) whether severely damaged or moderately damaged.

Table 2
Cytotoxicity of extracts from kudzu leaves treated with *Myrothecium verrucaria*

Kudzu extract[a] and toxin controls	% of plants infected	Harvesting time (day)	Approximate IC_{50} (mg/ml)[b]			
			H4TG	MDCK	NIH3T3	KA3IT
Control (0.2% SW)	0	1	35	35	40	20
M. verrucaria + 0.2% SW	50	1	20	15	30	15
Control (0.2% SW)	0	3	35	50	60	50
M. verrucaria + 0.2% SW	70	3	20	20	40	15
M. verrucaria + 0.2% SW	94	5	15	35	40	20
M. verrucaria + 0.2% SW	100	7	15	35	40	20
Control (0.2% SW)	0	10	15	40	75	30
M. verrucaria + 0.2% SW	100	10	20	20	40	20
M. verrucaria + 0.2% SW	100	14	7	5	10	5
Control (DMSO)	–	–	–	–	–	–
Verrucarin A	–	–	2 ng/ml	0.5 ng/ml	1.5 ng/ml	1 ng/ml
Roridin E	–	–	3.5 ng/ml	1.5 ng/ml	4 ng/ml	3.5 ng/ml
T-2 toxin	–	–	2 ng/ml	ND	ND	0.75 ng/ml

[a] SW = Silwet L-77, an organo-silicone surfactant (Union Carbide, Tarrytown, NY). Each sample was extracted with MeOH–chloroform, 1:1, v/v, dried, and redissolved in growth media containing 0.5% DMSO for cytotoxicity testing.
[b] The concentration of toxin which causes a 50% reduction in cell number after 5 days in culture. Cell lines used were H4TG, thioguanine-resistant rat hepatoma cells; MDCK, Madin-Darby canine kidney cells; NIH3T3; NIH Swiss mouse embryo fibroblasts, and KA31T, Kirsten strain of Moloney sarcoma virus-transformed 3T3 cells.

Table 3
Cytotoxicity of extracts from sicklepod treated with *Myrothecium verrucaria*

Sicklepod extract[a] and toxin controls	% of plants infected	Harvesting time (day)	Approximate IC_{50} (mg/ml)[b]			
			H4TG	MDCK	NIH3T3	KA3IT
Control (0.2% SW)	0	1	0.1	0.35	0.2	0.2
M. verrucaria + 0.2% SW	50	1	0.05	0.5	0.35	0.35
Control (0.2% SW)	0	3	0.1	0.35	0.2	0.2
M. verrucaria + 0.2% SW	70	3	0.05	0.5	0.35	0.35
M. verrucaria + 0.2% SW	95	5	0.05	0.35	0.35	0.35
M. verrucaria + 0.2% SW	100	7	0.15	0.75	0.5	0.2
Control (0.2% SW)	0	10	0.2	0.75	0.5	0.5
M. verrucaria + 0.2% SW	100	10	0.1	0.5	0.2	0.2
M. verrucaria + 0.2% SW	100	14	ND	ND	ND	ND
Control (DMSO)	–	–	–	–	–	–
Verrucarin A	–	–	2 ng/ml	1 ng/ml	2 ng/ml	1 ng/ml
Roridin E	–	–	4 ng/ml	2 ng/ml	4 ng/ml	4 ng/ml
T-2 toxin	–	–	2 ng/ml	ND	ND	1 ng/ml

[a] SW = Silwet L-77, an organo-silicone surfactant (Union Carbide, Tarrytown, NY). Each sample was extracted with MeOH–chloroform, 1:1, v/v, dried, and redissolved in growth media containing 0.5% DMSO for cytotoxicity testing.

[b] The concentration of toxin which causes a 50% reduction in cell number after 5 days in culture. Cell lines used were H4TG, thioguanine-resistant rat hepatoma cells; MDCK, Madin-Darby canine kidney cells; NIH3T3; NIH Swiss mouse embryo fibroblasts, and KA31T, Kirsten strain of Moloney sarcoma virus-transformed 3T3 cells.

Table 4
Cytotoxicity of extracts from soybean treated with *Myrothecium verrucaria*

Soybean extract[a] and toxin controls	% of plants infected	Harvesting time (day)	Approximate IC_{50} (mg/ml)[b]			
			H4TG	MDCK	NIH3T3	KA3IT
Control (0.2% SW)	0	1	0.2	1.0	0.75	0.75
M. verrucaria + 0.2% SW	50	1	0.5	1.5	1.0	0.5
Control (0.2% SW)	0	3	0.5	1.0	0.75	0.5
M. verrucaria + 0.2% SW	70	3	0.05	0.07	0.07	0.15
M. verrucaria + 0.2% SW	95	5	0.2	0.05	0.1	0.07
M. verrucaria + 0.2% SW	100	7	0.1	0.07	0.15	0.07
Control (0.2% SW)	0	10	0.2	1.5	0.5	1.0
M. verrucaria + 0.2% SW	100	10	0.05	0.01	0.04	0.02
M. verrucaria + 0.2% SW	100	14	0.2	0.15	0.2	0.15
Control (DMSO)	–	–	–	–	–	–
Verrucarin A	–	–	2 ng/ml	1 ng/ml	2 ng/ml	1 ng/ml
Roridin E	–	–	4 ng/ml	2 ng/ml	4 ng/ml	4 ng/ml
T-2 toxin	–	–	2 ng/ml	ND	ND	1 ng/ml

[a] SW = Silwet L-77, an organo-silicone surfactant (Union Carbide, Tarrytown, NY). Each sample was extracted with MeOH–chloroform, 1:1, v/v, dried, and redissolved in growth media containing 0.5% DMSO for cytotoxicity testing.

[b] The concentration of toxin which causes a 50% reduction in cell number after 5 days in culture. Cell lines used were H4TG, thioguanine-resistant rat hepatoma cells; MDCK, Madin-Darby canine kidney cells; NIH3T3; NIH Swiss mouse embryo fibroblasts, and KA31T, Kirsten strain of Moloney sarcoma virus-transformed 3T3 cells.

roridin E and T-2 toxin were very cytotoxic to the four cell lines used (Table 2). The IC_{50} of these toxins were $\leqslant 4$ ng/ml while the IC_{50} of kudzu plant tissues extracted with C:M, 1:1 was $\geqslant 5$ mg/ml.

These results demonstrate that *M. verrucaria* is an effective pathogen against kudzu and sicklepod, causing disease on all kudzu plants after 96 h. Therefore, *M. verrucaria* shows good potential for control of these noxious weeds. The isolate of *M. verrucaria* used in this study was found to produce large amounts of macrocyclic trichothecenes in culture. These toxins, specifically verrucarin A and roridin E, have proven to be very toxic to plants and animals (Cutler and Jarvis, 1985; Bean et al., 1988; Jarvis and Acierto, 1989). Toxin production has caused concern about the safety for animals and humans of using *M. verrucaria* in biological control. Although the amounts and numbers of toxins produced varied with substrate, none of these toxins were detected in the tissues of treated kudzu plants. To ensure that kudzu was not unique in this, soybean and sicklepod, which are in the same family (Leguminosae) as kudzu, were also studied. No toxins were isolated from *M. verrucaria*-treated sicklepod or soybean. These results are in agreement with previous reports (Bean et

al., 1984; Kuti et al., 1987) on watermelon, muskmelon and cucumber (all from the family "Cucurbitaceae") infected with *Myrothecium* species. These investigators were also unable to detect macrocyclic trichothecenes in infected tissues. However, there are numerous reports of macrocyclic trichothecenes being produced by fungal endophytes in shrubs known as *Baccharis* spp. (Busam and Habermehl, 1982; Jarvis, 1992; Jarvis et al., 1996; Rizzo et al., 1997). These toxins may have been made by the plants using genes for trichothecene biosynthetic enzymes acquired from fungi, but it is also possible that the toxins may be metabolites of trichothecenes produced by contaminating fungi. The failure to find in treated tissue either intact trichothecenes by HPLC, or toxic metabolites by cytotoxicity tests in the present study supports the case for using *M. verrucaria* as a biological control agent. The results support animal feeding trials as the appropriate next step in evaluating safety. Additional studies are needed to quantitate the extent of colonization of kudzu tissues after treatment with *M. verrucaria* conidia preparations. The results obtained to date provide evidence for both safety and efficacy for the use of *M. verrucaria* in biological control of kudzu and other noxious weeds.

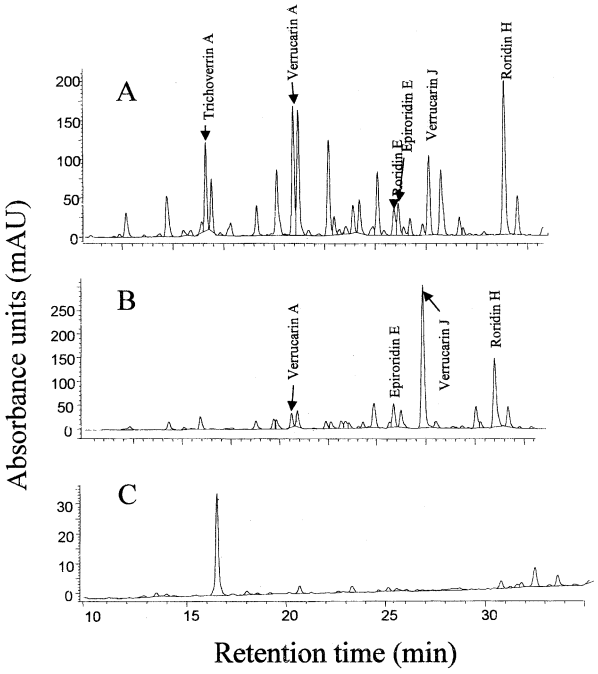

Fig. 1. HPLC chromatographs of extracts from (A) rice culture medium infected with *Myrothecium verrucaria* spores, (B) rice culture medium infected with *M. verrucaria* fungal mycelia, and (C) kudzu plant tissue treated with *M. verrucaria* conidia. Note the presence of various macrocyclic trichothecenes in the extracts of moldy rice culture medium infected with spores or mycelia. No toxins were detected in the extract of kudzu tissues treated with this fungus.

3. Experimental

3.1. Mycotoxin production on rice culture media

Converted long-grain enriched parboiled rice (200 g; Uncle Ben's Inc., Greenville, MS) was mixed with 120 ml of distilled water in one liter flasks, and allowed to stand about 1 h until the water was absorbed. The flasks were shaken to uniformly distribute the moist substrates, closed with light cotton stoppers and autoclaved 60 min at 15 lb pressure. Immediately after autoclaving, the flasks were shaken vigorously to break up clumps. They were allowed to stand 24 h and the autoclaving and shaking process was repeated. The flasks were inoculated when cool with *M. verrucaria* (International Mycological Institute, Bakeham Lane, Egham, Surrey, UK; holding IMI No. 361690) maintained in stock cultures on sterile soil or silica gel in small test tubes. The flasks were incubated for 3 to 4 weeks either in the laboratory at 22–24°C, or in an incubator at 28°C with 12 h light intervals. Daily shaking for the first few days was used to enable the fungus to penetrate the rice uniformly. The fungus-infested rice was removed from the flasks and air dried on a screen-bottomed tray. Five grams of mouldy rice were ground to the consistency of flour using a Stein Mill grinder (Fred Stein Laboratories, Inc., Atchison, KS), and samples taken for chemical analyses (Abbas et al., 1984; 1988). All operations with fungal cultures and spores were carried out in a vented hood to prevent exposure to investigators and facilities.

3.2. Mycotoxin standards

Verrucarin A and J, roridin E and isororidin E were purchased in analytically pure form from Sigma Chemical Co., St Louis, MO. An additional 55 mycotoxin standards were prepared in the laboratory of B.B. Jarvis, Department of Biochemistry and Chemistry, University of Maryland, College Park, MD (Jarvis and Wang, 1999).

3.3. Extraction and determination of mycotoxins

Dried, ground moldy rice cultures (5 g) of *M. verrucaria* were extracted with methanol:chloroform, 1:1 (v/v) at a ratio of 1 g mouldy rice to 5 ml extracting solvent, using ultrasound agitation for 1 h, or homogenization for 3 to 5 min with a Brinkmann Polytron PT 3000 at 27.8×1000 rpm. The flask and contents were allowed to sit overnight (4°C) before filtration and concentration of the organic extract. The concentrated organic extract was dissolved in a minimum amount of methylene chloride and applied to a polyethyleneimine column (Jarvis, 1992). Elution of the column with methylene chloride gave fractions which were analyzed by reversed phase HPLC on a HP-1100 system using an 150×2.0 mm, 3 μ C-18 column and a UV–vis diode array detector at 260

nm (Hinkley and Jarvis, 2000). Samples (5 μl) were eluted with gradients of 25 to 100% acetonitrile in water as the mobile phase. Peak assignments were made by a combination of retention times and UV spectra compared to purified trichothecene standards. Extraction efficiencies of 90–95% were obtained in plant tissues for the various macrocyclic trichothecenes.

3.4. Mycotoxin production in liquid media

This study evaluated *M. verrucaria* fermentation in two liquid media, cornsteep medium according to the method of Jarvis et al. (1986), and cornmeal–soyflour medium according to the method of Walker and Riley (1982). Fermentation in cornsteep medium was carried out in two stages. Five inoculum cultures were prepared in Erlenmeyer flasks (500 ml) each containing 100 ml of cornsteep medium (15.6 g of glucose and 10 ml of cornsteep liquor per l water) autoclaved at 121°C for 20 min. The flasks were inoculated with one or two 1 cm^2 pieces of PDA agar (Difco) medium from plates carrying *M. verrucaria* mycelium and conidia (spores), and shaken at 150 rpm at 28°C for 3 days to produce the inoculum for the production phase. Five Erlenmeyer flasks (4 l) each containing 1 l of production medium (1.0 g $NH_4H_2PO_4$, 3.0 g K_2HPO_4, 5.0 g NaCl, 0.2 g $MgSO_4$–$7H_2O$, 40 g sucrose and 10 g glycerol per l of water) were autoclaved for 30 min at 121°C, and one inoculum culture was poured into each flask. The cultures were incubated with shaking (150 rpm, 28°C) for 7 days, and the mycelium and the fermentation beer harvested separately by centrifuging for 10 min at 10,000 rpm and filtering through glass wool. The mycelium was soaked in acetone three times and homogenized at high speed using Polytron PT 3000, for 3 to 5 min and centrifuged. All the acetone extracts were combined and the acetone removed on a rotary evaporator. The aqueous concentrate was extracted with ethyl acetate three times. Ethyl acetate extracts were combined and dried over Na_2SO_4, filtered and evaporated. The fermentation beer was extracted with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4, filtered and evaporated. The material obtained from the mycelium and the fermentation beer were combined to give 9 g of total extract. The toxin content of these extracts was determined by HPLC as described above.

The second medium consisted of cornmeal (0.44 g), soyflour (3.75 g), sucrose (7.5 g) and calcium carbonate (0.75 g) in 600 ml of deionized water. Aliquots of 50 ml were placed in 300 ml Erlenmeyer flasks and autoclaved. The flasks were inoculated with one or two 1 cm^2 pieces of PDA agar medium from plates carrying *M. verrucaria* mycelium and conidia (spores), and incubated with shaking at 150 rpm for 7 to 10 days at 28°C. When the fungal biomass formed, the same procedure was followed for extraction and determination of toxin content as described above for cornsteep medium.

3.5. Determination of mycotoxins in fungal spores and mycelia

Spores and mycelia of *M. verrucaria* for this study were produced on Petri dishes (9 cm inside diameter) containing 20 to 25 ml PDA as described in detail previously (Abbas and Riley, 1996). After inoculation Petri dishes were incubated 12 days at 28°C with 12 h light and 12 h dark cycles. Spores and mycelia were harvested from agar surfaces using a plastic scraper and transferred to a sterile beaker. Five hundred grams (wet weight) of mycelium was obtained from 500 Petri dishes. The mycelium sample was mixed well, and an aliquot of 5 g was taken for extraction and mycotoxin analysis by the same procedure as described above for mycelium from liquid cultures.

3.6. Determination of mycotoxin from infected plant tissues

Kudzu (*Pueraria montana*), sicklepod [*Senna obtusifolia* (L.) Irwin and Barneby], and soybean [*Glycine max* (L.) Merr.] were used in this study. Kudzu seedlings (2 to 3 true leaf stage) were grown from seed (Adams-Briscoe Seed Co., Jackson, GA 30233) in 30×60 cm plastic trays containing a 1:1 commercial potting mix (Jiffy mix®): soil combination supplemented with a controlled-release 13:13:13 (N:P:K) fertilizer. Sicklepod and soybean seedlings were grown from seed (collected locally, Stoneville, MS 38776) in the field during the growing season. All plants were kept outdoors. Seedlings at the 1st to 3rd leaf growth stage were inoculated by aerosol sprayers until foliage was fully wetted with 2×10^7 conidia/ml suspended in 0.2% aqueous Silwet L-77 surfactant. Control (non-infected) groups received 0.2% Silwet L-77 surfactant only. Following inoculation, infected and non-infected plants were sampled at the following times: 0, 1, 2, 3, 4, 5, 7, 10, and 14 days. Each sample was divided based on disease symptoms into two groups: (i) severely infected (total necrosis, brown dry tissues, dead) and (ii) moderately or partially infected tissue (minimal necrosis, green, healthy tissue to partially-infected leaves). Also, root samples were kept separate from the above-ground samples for each treatment and species. Root samples were washed with water to remove dirt before storage inside plastic bags. Samples were brought to the laboratory and each sample was cut into small pieces prior to processing for analyses. Half of each sample was frozen in liquid nitrogen and lyophilized. The other halves of the samples were put into paper bags and dried in an oven at 70–75 °C for 72 h. Each dried sample was ground to the consistency of powder using a Stein Mill grinder before analysis. All samples were stored at −20 °C. Aliquots of 5 g were taken for mycotoxin analyses using the same procedure as described above.

3.7. Cytotoxicity assay

The mammalian permanent cell line 3T3 Swiss mouse fibroblasts (strain NIH3T3) was obtained from S. Aaronson, National Cancer Institute, Bethesda, MD, USA, and KA31T was obtained from R. Pollack, Columbia University, New York, NY. Rat hepatoma cell line H4TG and dog kidney cell line MDCK were purchased from the American Type Culture Collection, Rockville, MD, USA. The cells were cultured and the cytotoxicity assays were conducted as described previously (Abbas et al., 1995; Shier et al., 1991).

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