

## **Enzyme-Linked Immunosorbent Assay Detection of Trichothecenes Produced by the Bioherbicide *Myrothecium verrucaria* in Cell Cultures, Extracts, and Plant Tissues**

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**Abstract:** A rapid technique for trichothecene detection was needed in screening tests of the potential bioherbicide *Myrothecium verrucaria* (MV), in order to select strains, mutants, or formulations that were void of or that possessed low amounts of these undesirable mycotoxin compounds. Commercially available enzyme-linked immunosorbent assay (ELISA) plates for trichothecene detection, possessing cross-reactivity with several trichothecene mycotoxins (e.g., verrucarins A, and J, roridin A, L-2, E, and H), were tested for their ability to detect trichothecenes produced by a strain of *Myrothecium verrucaria* (MV) in cell cultures, in plant tissues (hemp sesbania and kudzu) treated with purified roridin A, or ethyl acetate fractions of MV cultures. Evaluations of ELISA assays showed linear responses for standards of verrucarins A and roridin A over a concentration range of 0.2 to 20 ppb. Ethyl acetate or aqueous extractions were used to obtain samples from MV cultures and plant tissues for testing. Trichothecenes were detected in conidia and mycelia of MV, and in agar upon which wild-type MV was grown, indicating secretion into the growth media. Two MV sectors (morphological variants of wild type) also tested positive for trichothecenes. Purified roridin A and concentrated extracts containing trichothecenes from MV spore cultures exhibited phytotoxicity (growth inhibition or necrosis) when applied to excised shoots of hemp sesbania seedlings and intact kudzu leaf tissues. Evidence of some translocation of trichothecenes from the

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application point in kudzu was found, but translocation to the upper shoot portion of hemp sesbania was not detected at the lowest limit of detection in this assay (0.14 ppb). This assay is also being employed to identify induced mutants and/or other naturally occurring sectors deficient in trichothecene mycotoxin production. Results indicated that ELISA is a sensitive and rapid assay method to quantify trichothecenes produced by this bioherbicidal fungus and in certain plant tissues treated with trichothecenes.

**Keywords:** Bioherbicide, biological weed control, ELISA, hemp sesbania, kudzu, mycoherbicide, mycotoxin, trichothecene

## INTRODUCTION

Bioherbicides are phytopathogenic microorganisms or microbial phytotoxins useful for weed control (Hoagland 2001; Charudattan 2005; Weaver et al. 2007). Virulence factors (lytic enzymes, phytotoxins, elicitors, etc.) for several bioherbicidal microorganisms have been elucidated, and in some cases phytopathogenic taxa have been associated with the production of compounds that are toxic to mammals (Vey, Hoagland, and Butt 2001; Goettel et al. 2001; Hoagland, Boyette, and Weaver 2007). Several isolates of the fungus [*Myrothecium verrucaria* (Alb. and Schwein.) Ditmar:Fr.] (MV) possess bioherbicidal activity, but many members of this genus produce mammalian-sensitive mycotoxins (e.g., macrocyclic trichothecenes). We are studying an MV isolate (IMI acc. no. 361690) originally isolated from sicklepod (*Senna obtusifolia* L.) that exhibits bioherbicidal activity against kudzu [*Pueraria lobata* (Willd.) Ohwi] and several other weeds (Walker and Tilley 1997; Boyette, Walker, and Abbas 2002; Anderson and Hallett 2004; Hoagland, Boyette, and Weaver 2007). A U.S. patent was issued for the use of this MV strain as a bioherbicide for kudzu control (Boyette, Walker, and Abbas 2001). Although *M. verrucaria* (IMI 361690) can effectively control several species of weeds, the fact that this strain produces mycotoxins has hindered U.S. Environmental Protection Agency (USEPA) registration of this bioherbicide. Subsequently there is also a hesitancy of industry to develop this fungal strain as a commercial bioherbicide. Kudzu, a perennial leguminous vine native to eastern Asia, was introduced into the United States in the late 1800s (McKee and Stephens 1943). It now occurs from Florida to New York, westward to central Oklahoma and Texas, with the heaviest infestations in Alabama, Georgia, and Mississippi (Miller 1985). In a 1993 U.S. Congressional Report, kudzu was cited as one of the most harmful nonindigenous plants in the United States. Kudzu is also important because it has been found to serve as an alternate overwintering host for Asian soybean rust (*Phakopsora*

*pachyrhizi* Syd. & P.) in the southern United States (Plant Health Initiative, North Central Soybean Research Program, 2007 [http://www.planthealth.info/rust\\_basics.htm](http://www.planthealth.info/rust_basics.htm)). Kudzu is very difficult to control with conventional chemical herbicides, and this weed continues to expand its range each year. MV also has potent bioherbicidal activity on another leguminous weed, hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. Ex A.W. Hill] (Walker and Tilley 1997; Boyette et al. 2007), a very troublesome weed in soybeans [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.), and rice (*Oryza sativa* L.) in the southern United States (Lorenzi and Jeffery 1987; King and Purcell 1997). Additionally, the invasive weeds redvine [*Brunnichia ovata* (Walt.) Shinnery] and trumpet creeper [*Campsis radicans* (L.) Seem. Ex Bureau] are moderately susceptible to MV, but not at levels that provide acceptable weed control (Boyette, Reddy, and Hoagland 2006).

Improved performance of some bioherbicides, including MV, can occur through chemical synergism, especially with the herbicide glyphosate (Hoagland 2001; Boyette, Reddy, and Hoagland 2006). During efforts to develop MV as a bioherbicide, it was discovered that this isolate produced certain verrucarins (trichothecenes), which are mycotoxins that are highly toxic to mammals (Sudakin 2003). Production of such mycotoxins could hinder registration of such microbes for commercial use as bioherbicides, although a strain of *Myrothecium verrucaria* has been registered as a nematicide, and toxicity tests of this product have proven negative on some invertebrates (Warrior et al. 1999). Furthermore, a *Myrothecium verrucaria* isolate from leafy spurge (*Euphorbia esula* L.) has a host range that differs from that of the strain we are studying; however, it also produces trichothecenes (Yang and Jong 1995a, 1995b; Millhollon et al. 2003).

It became clear in our studies that a rapid technique for trichothecene detection would be valuable for screening MV strains, mutants, and formulations as potential bioherbicides that were void of or possessed low amounts of trichothecenes. Analytical methods for mycotoxin detection have improved over the past years. Chromatographic methods such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) are the most accurate quantification systems, but immunological methods, especially enzyme-linked immunosorbent assays (ELISAs) are widely used and they usually do not require extensive sample purification. The ELISA methodology has been adapted in a wide variety of instances to determine low concentrations of various pesticides (herbicides, insecticides, and fungicides) in soils, water, and plant and animal tissues (Hall, Deschamps, and McDermott 1990). Trichothecenes in cereal grains have been monitored by thin-layer chromatography (TLC), HPLC, ELISA, and GC using fluorescence or electrochemical detection (Tanaka et al. 1985; Trucksess et al. 1987;

Lauren and Greenhalgh 1987; Morgan 1989). Macrocyclic trichothecenes have also been detected on small airborne particulates using ELISA (Brasel et al. 2005).

ELISA test kits also offer high-throughput assays with low sample volume requirements and rapid processing times (< 1 h) for multiple samples. Although the antibodies have high specificity and sensitivity to their mycotoxin target molecules, some compounds with similar chemical moieties may also react with the antibodies (Binder 2007).

To eliminate or reduce trichothecene levels via cultural, mutagenesis, sector\* selection, chemical regulation, and/or formulation, we required a rapid, sensitive, and specific assay for these compounds in cultures of MV isolates and strains. Therefore, the objectives of these experiments were to examine ELISA methodology for detection and quantification of trichothecenes in MV cultures, strains, and sectors and for utility in monitoring absorption, translocation, and extractability of trichothecenes in kudzu and hemp sesbania tissues. The phytotoxicity of roridin A and an extract of MV containing trichothecenes in these plants was also investigated.

## MATERIALS AND METHODS

### Chemical Sources

The trichothecenes, roridin A and verrucarin A, were analytically pure preparations obtained from Sigma Chemical Company, St. Louis, MO. All other laboratory chemicals were of reagent-grade quality or higher. The surfactant Silwet L-77 was obtained from Osi Specialties, Inc., Charlotte, NC

### Inoculum Production

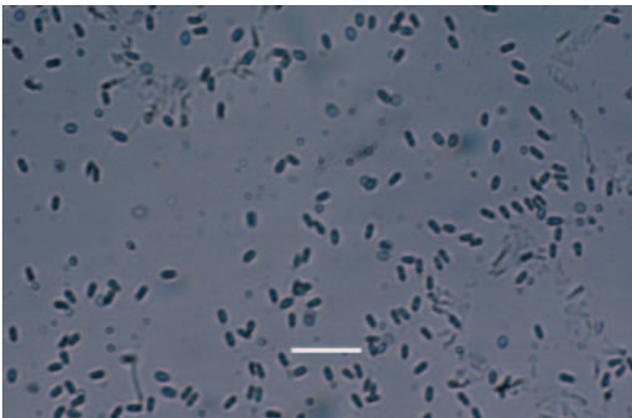
The isolate of MV used in these tests was IMI 361690 (International Mycological Institute, Bakeham Lane, Egham, Surrey, U.K.). Inoculum of MV (Figure 1) was produced in Petri® dishes containing Difco potato dextrose agar (PDA) (DIFCO Laboratories, Detroit, MI). Conidia of this MV isolate are relatively small and have been reported to range as

\* A sector refers to the spontaneous expression of different morphological traits of the wild-type organism during radial growth in Petri® dish culture, resulting in an observable different culture phenotype within the radial growth pattern. Such an event causes the appearance of a sector of contrasting fungal growth within that culture plate.

6.5–8 × 2.0–3.5 μm (Walker and Tilley 1997). Agar surfaces were flooded with an MV conidia suspension of  $6 \times 10^6$  conidia. Plates were then inverted on open-mesh wire shelves and incubated (25 °C, 5 days) in fluorescent-lighted incubators. Conidia were rinsed from plates with sterile, distilled H<sub>2</sub>O and adjusted to desired concentrations with H<sub>2</sub>O. Conidial concentrations were determined using a hemacytometer. Sectors of MV were manipulated in a similar manner. Conidia of sectors and/or the wild-type strain of MV were extracted or inoculated onto solid substrate (rice grains) for growth and trichothecene analysis as described later.

### Fungal Growth on Solid Substrate (Rice Grains)

Long-grain, converted, enriched rice grains (200 g) were mixed with 120 mL H<sub>2</sub>O in 1-L flasks and allowed to stand for approximately 1 h until the water was absorbed. Flasks were plugged with cotton and autoclaved 60 min. After cooling, the flasks were shaken to break up clumps, allowed to stand 24 h, and then autoclaved, shaken again, and cooled. The flasks were inoculated with MV spores (10 mL at  $2.0 \times 10^7$  mL<sup>-1</sup>) and incubated 3 to 4 weeks at 22 °C with a 12-h photoperiod (approximately 200 μmol m<sup>-2</sup> s<sup>-1</sup>, PAR). Daily shaking of the flasks for the first several days assured uniform distribution/penetration of the fungus into the rice grains. At the end of the incubation, the fungal–rice mixture was spread onto screen-bottomed trays and air dried. Dried samples were ground to a flour-like consistency using a mill grinder (Stein Mill, model M-2; F. Stein Laboratories, Inc., Atchison, KS) housed in a biosafety cabinet. Crude preparations were made by stirring various



**Figure 1.** *Myrothecium verrucaria* conidia, harvested from 7-day-old potato dextrose agar culture. Bar = 10 microns.

amounts of this MV–rice mixture in 100 mL distilled water for 15 min. Extracts were then clarified by centrifugation ( $12,000 \times g$ , 10 min), and aliquots of the supernatants were tested for trichothecene content using ELISA. A minimum of three replications of each MV–rice mixture ratio were extracted, and the experiments were repeated in time.

### Plant Propagation

Kudzu seeds were purchased from Adams Briscoe Seed Co., Jackson, GA. Seedlings were grown from seeds in pots [1:1 commercial potting mix–soil with 13:13:13 nitrogen (N)–phosphorus (P)–potassium (K) fertilizer] in a greenhouse (28 to 32 °C; 40–60% RH; 14 h photoperiod at 1600 to 1800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , PAR at midday). Hemp sesbania seeds were obtained from weed nursery field plots grown at the SWSRU, Stoneville, MS. The seeds were mechanically scarified and grown hydroponically under continuous darkness in a growth chamber as outlined elsewhere (Hoagland 1995).

### Plant Treatments and Extraction

Uniform greenhouse-grown kudzu seedlings in the fourth trifoliolate growth stage were exposed to roridin A or an extract of MV cultures containing trichothecenes by spotting these trichothecene solutions prepared in a dilute surfactant (0.02%, v/v Silwet L-77) with a microliter pipette to a single leaflet of the pair of trifoliolate leaflets of the youngest trifoliolate. Control plant leaflets received 0.02% aqueous Silwet L-77. Treated seedlings were then placed in a greenhouse, and leaflet harvests were made at 72 h after treatment. Plant trifoliolate leaflets (single treated leaflet of the pair of trifoliolate leaflets, the opposite leaflet, and the terminal leaflet) were visually examined for phytotoxic effects and then excised, weighed, and homogenized in 1.0 mL phosphate buffered saline [0.01 M, pH 7.4; sodium chloride (NaCl) = 0.138 M; potassium chloride (KCl) = 0.0027 M] using handheld glass tissue homogenizers. Extracts were clarified by centrifugation at  $12,000 \times g$  for 10 min. Supernatants (50  $\mu\text{L}$  each) were assayed directly for trichothecene content via ELISA. A minimum of three replications consisting of four plants per replicate of each treatment were extracted and analyzed.

Uniform hemp sesbania seedlings (grown as described previously) were selected and their roots excised with a scalpel to obtain plant shoots 45 mm in length. The cut ends of the plants were placed in microcentrifuge tubes containing solutions of 1.0 mL roridin A at several concentrations or the MV trichothecene preparation at several concentrations, and then

placed in darkness in a growth chamber to monitor possible growth or phytotoxicity effects over a 48-h time course. Control seedling shoots received H<sub>2</sub>O. At termination, the length of each excised plant was measured and then divided into two parts (i.e., treated end = 35 mm from cut end upward and the remaining upper shoot). These two segments were ground and extracted separately in phosphate buffered saline and then analyzed for trichothecene content as described previously for kudzu seedling extracts. A minimum of three replications of each treatment consisting of three to four plant shoot segments per replicate were extracted and tested, and the experiments were repeated in time.

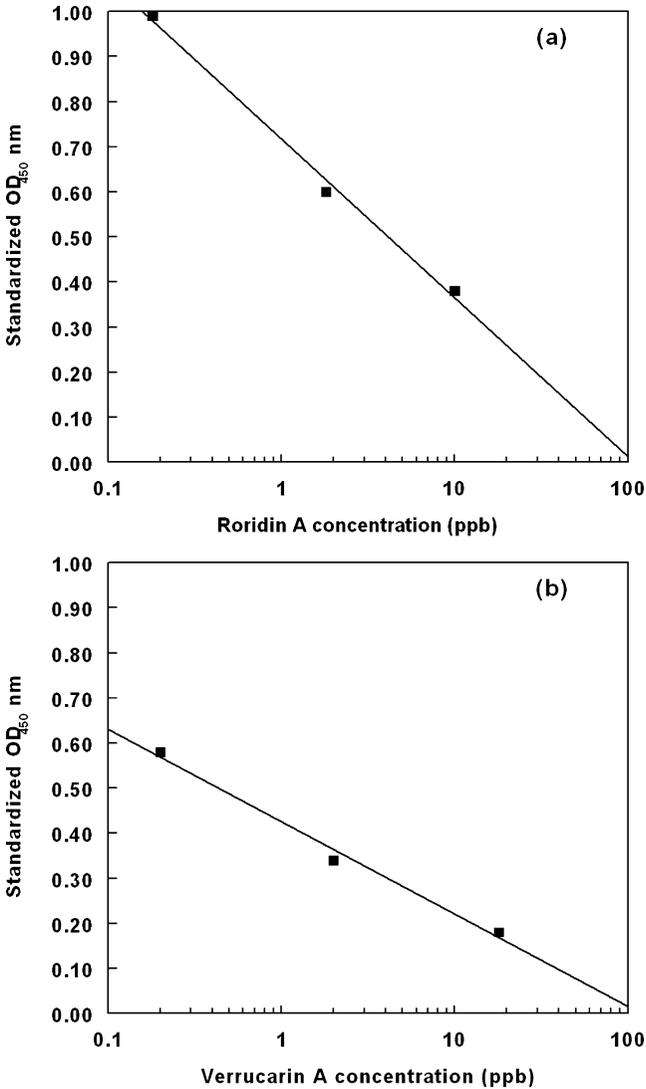
### ELISA Well Plates

The ELISA plates were purchased from EnviroLogix, Portland, ME, and the assays were conducted according to manufacturer's protocols. This assay kit is specific for trichothecenes; however, the antibody cross-reacts with several trichothecenes (e.g., verrucarin A and J, roridin A, L-2, E, and H) as discussed in the manufacturer's information data sheets for this product. Hence, the values obtained reflect the total trichothecene content in each sample. Trichothecenes in the test samples compete with horseradish peroxidase-labeled satratoxin for antibody binding sites coated on the well surface. Developed plates were read on a  $\mu$ Quant plate reader (BioTek Industries, Inc., Winooski, VT.) at a wavelength of 450 nm. All test samples and standards were run in triplicate, and the experiments were repeated in time.

## RESULTS AND DISCUSSION

### Standardization and Cross-reactivity

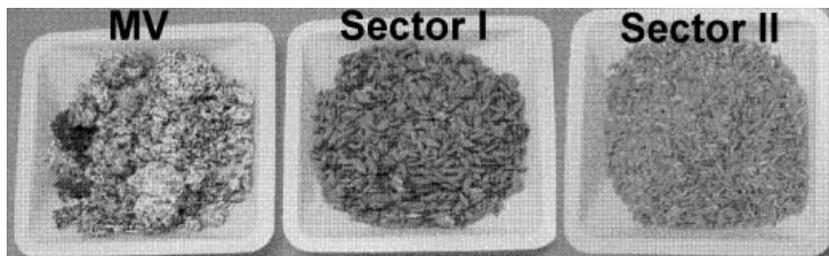
Preliminary concentration versus optical density response was initiated using two high-purity trichothecenes. Standard curves of roridin A and verrucarin A concentration versus o.d. at 450 nm indicated linear relationships for both compounds in a range of 0.10 to 100 ppb when analyzed via ELISA (Figure 2). There was nearly twice the reactivity to roridin A compared to verrucarin A, because the antibody was raised against roridin A. As per the manufacturer's description of this ELISA product, there is cross-reactivity among various macrocyclic trichothecenes, this kit cannot distinguish among the trichothecenes, and these compounds are detected at differing sensitivity levels. Nevertheless, this assay can provide a quantitative measure of trichothecenes and closely related molecules present in various cultures or tissues.



**Figure 2.** Standard curve of roridin A (a) and verrucarin A (b) concentration versus o.d. at 450 nm as measured via ELISA. The linear relationship of o.d. versus concentration is best described by the equations  $Y = 0.717 - 0.352 X$ ,  $R^2 = 0.98$ , and  $Y = 0.425 - 0.205 X$ ,  $R^2 = 0.98$ , for roridin A and verrucarin A, respectively.

### Mycotoxin Production and Extraction

In preliminary testing previously reported (Hoagland, Weaver, and Boyette 2005), trichothecene production occurred in several sectors of MV that were isolated from the wild-type MV cultured on PDA plates

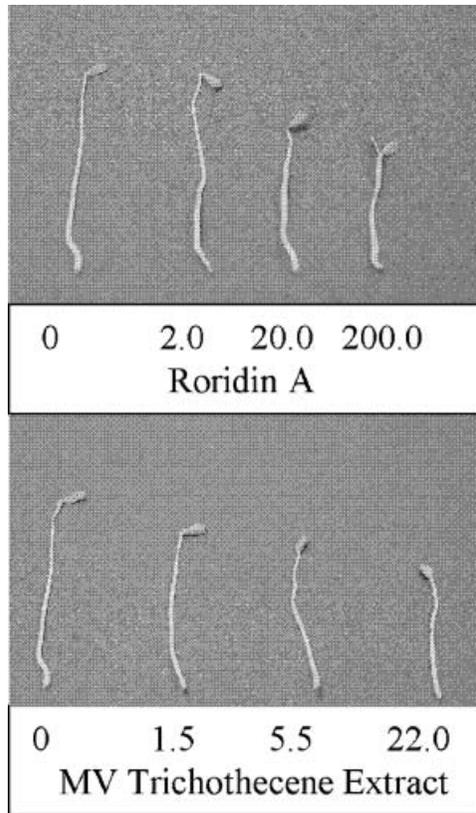


**Figure 3.** Examples of MV sector isolates cultured on PDA and grown on solid substrate (rice grains).

and propagated on rice grain as a solid substrate. These tests were repeated and verified in these present studies (data not shown). Aliquots of solid substrate (rice) extracts of MV and two MV sectors (see Figure 3) did not differ significantly from each other with respect to total trichothecene content as determined via ELISA (data not shown). Thus, although these two sectors were quite different morphologically, their trichothecene biosynthetic ability was not appreciably altered compared to the parent MV culture. In other extraction tests using ethyl acetate partitioning, MV spores and the PDA on which MV was cultured were also examined for trichothecene levels. Trichothecene levels in the ethyl acetate fraction of MV cells were greater than those occurring in the agar (data not shown). This indicates that trichothecenes are released from the fungal cells with migration into the growth media (agar). These tests on MV and MV sectors were not quantitative but were only tested qualitatively for the presence or absence of trichothecenes.

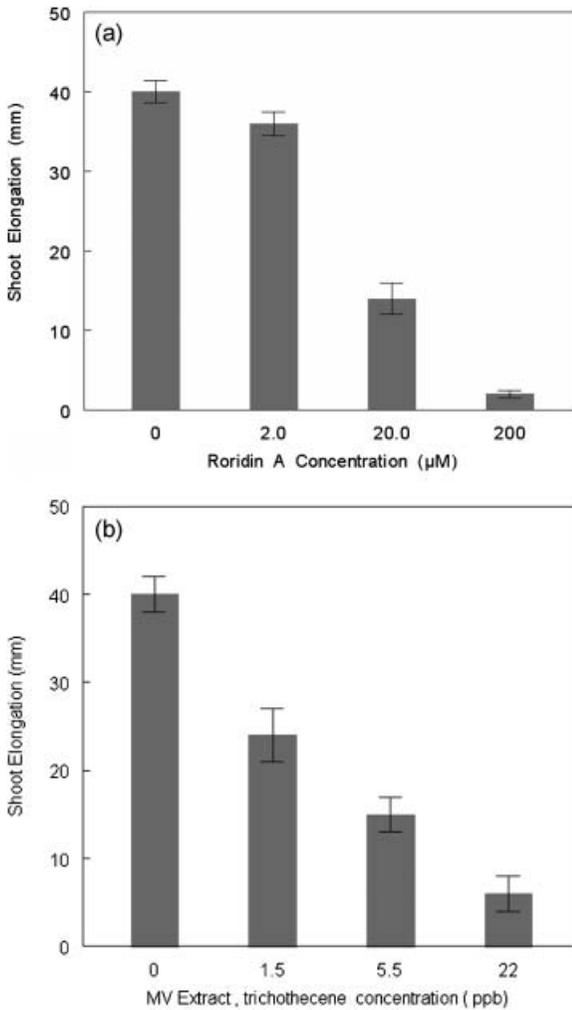
### **Expression of Trichothecene Phytotoxicity in Hemp Sesbania and Kudzu Seedlings**

Roridin A and the MV trichothecene extract preparation both exhibited concentration-dependent growth reduction (shoot elongation) in dark-grown, excised hemp sesbania shoots (Figure 4). Plots of actual elongation (mm) from the inception of treatment when all excised plants were 45 mm in length indicated that both solutions curtailed elongation at their highest application rate (Figure 5). The MV trichothecene preparation exhibited more growth reduction, but it had a higher total trichothecene concentration than the roridin A alone treatment. This may suggest that roridin A is more phytotoxic to hemp sesbania than the overall mixture of trichothecenes contained in the MV extract. It also appears that proportionally more roridin A in the high purity standard solution than trichothecenes in the MV extract was taken up by hemp



**Figure 4.** Visual effects of roridin A and the MV extract at several concentrations on dark-grown, excised hemp sesbania shoots, 48 h after feeding in continuous darkness.

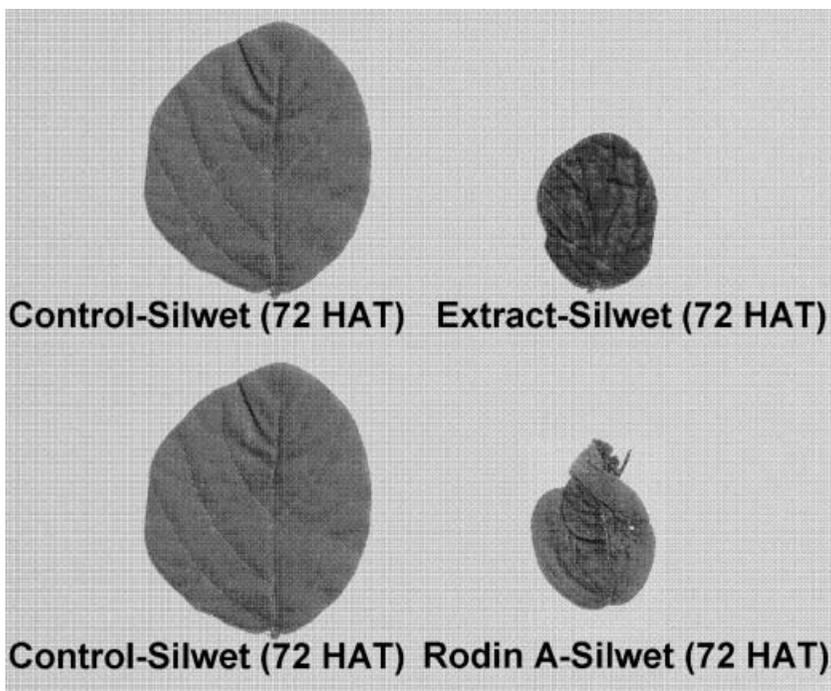
sesbania shoot segments. Phytotoxic symptoms of kudzu trifoliolate leaf pairs were evident 72 h after treatment with roridin A and the MV extract (Figure 6). Both chemical treatments caused severe necrosis in the treated leaflet of kudzu. Growth reduction of the treated leaflet of the trifoliolate pair in the roridin A (50 ppb) and MV extract (1500 ppb) treatments were substantially lower compared to the opposite leaflet (i.e., a 30% and 20% reduction in fresh weight, respectively). Other studies have reported the phytotoxicity of trichothecenes on various plant species (Alexander, McCormick, and Ziegenhorn 1999; Abbas et al. 2002; Proctor et al. 2002). Our results indicate that a trichothecene (roridin A) is phytotoxic when the pure compound is administered to hemp sesbania and kudzu tissues. However, further investigations will be needed to definitively implicate a role for trichothecenes as MV virulence factors in hemp sesbania and kudzu as has been suggested for some *Fusarium* pathogens that act on other plant species (Harris et al. 1999; Proctor et al. 2002).



**Figure 5.** Growth effects [elongation (mm)] of (a) roridin A and (b) the MV extract at several concentrations on dark-grown, excised hemp sesbania shoots, 48 h after continuous feeding in continuous darkness. Data presented is elongation (mm) that the seedlings grew during the 48-h treatment period. Error bars represent  $\pm 1$  S.E.M.

### Absorption and Translocation of Trichothecenes in Plant Tissues

ELISA analysis of hemp sesbania (Table 1) and kudzu (Table 2) tissues for the uptake and possible translocation of trichothecenes indicated that the treated areas retained substantial amounts of the treatment chemicals, whereas only low amounts of trichothecenes were found in tissues of



**Figure 6.** Visual effects of roridin A and the MV extract at several concentrations on greenhouse-grown kudzu seedlings, 72h after foliar application to a single leaflet of the trifoliolate leaflet pair of the youngest trifoliolate.

**Table 1.** Trichothecene content in dark-grown hemp sesbania seedling tissues treated with roridin A or MV extract

Treatment	Trichothecene content (ppb) <sup>a</sup>	
	Treated shoot segment	Upper shoot segment
Roridin A		
0	0	nd <sup>b</sup>
1 ppb	0.9 ± 0.2	nd
10 ppb	12.1 ± 1.3	nd
100 ppb	22.3 ± 2.1	nd
MV extract		
0	0	nd
15 ppb	0.06 ± 0.01	nd
75 ppb	4.4 ± 0.9	nd
600 ppb	16.2 ± 1.9	nd

<sup>a</sup>Trichothecene concentration determined in 50 µL of tissue extract as determined by ELISA. Values are means of three replications, ± 1 S.E.

<sup>b</sup>nd = none detectable.

**Table 2.** Trichothecene content in kudzu seedling tissues treated with roridin A or MV extract

Treatment	Trichothecene content (ppb) <sup>a</sup>		
	Treated leaflet	Opposite leaflet	Terminal leaflet
Roridin A			
0	0	0	0
50 ppb	17 ± 1.4	0.10 ± 0.1	0.30 ±
MV extract			
0	0	0	0
1500 ppb	12 ± 1.1	> .10	0

<sup>a</sup>Trichothecene content in 50 µL of tissue extract as determined by ELISA. Values are means of three replications, ± 1 S.E.

these plants adjacent to the chemical application sites. This suggests low mobility of these compounds in these tissues, possible metabolism of the mycotoxins, or that dilution factors during extraction were too large. We did not examine trichothecene persistence in these plant tissues, but this important aspect will be investigated in future research. Other studies using HPLC have indicated a lack of detectable trichothecenes in plant tissues of several weeds [kudzu, hemp sesbania, and soybean (*Glycine max*)] when treated with MV spore formulations (Abbas et al. 2001). However, trichothecene levels in those formulations may have been very low (less than detection levels based on amount applied and/or sample extraction dilutions), and those studies tested plants outdoors at various times, up to 14 days after treatment. Thus, harsher and more diverse environmental factors, coupled with longer time courses, may have reduced or curtailed production of trichothecenes by MV. Furthermore, decreased absorption/translocation by plant tissues, and/or possibly increased degradation or metabolism of these compounds on or in those plant tissues might have occurred under field conditions. We are not aware of other studies that have specifically investigated translocation of trichothecenes in plants using ELISA.

## SUMMARY AND CONCLUSIONS

The MV (wild type) and some MV sectors that formed in Petri<sup>®</sup> dish cultures or that were grown on rice grain tested positive for trichothecenes using this ELISA method. In these initial tests, which are part of a large, ongoing project, all samples of MV and several mutants thus far tested were found to produce trichothecenes. In our mutant induction and selection studies, or through nutrient culture and spray formulation development, we hope to obtain a highly virulent MV

isolate or preparation that is void of or that produces or contains only very low amounts of these mycotoxins.

Our results also show that ELISA can be used to detect and quantify trichothecene levels in plant tissues when these compounds are applied to foliage or fed through roots. In greenhouse tests, some translocation of trichothecenes (high purity roridin A or trichothecenes for the MV extract) from the point of application occurred in intact kudzu seedlings, but translocation in excised hemp sesbania seedlings was not demonstrated. This apparent lack of detection of trichothecenes in the upper portion of excised hemp sesbania shoots could simply be due to too much dilution during the tissue sample preparation. As with many preparation techniques, sample size is often a limiting factor, and extraction volumes must be adequate to achieve uniform and homogeneous grinding, and optimal extraction efficiency. Another consideration concerning extraction of compounds from plant tissues is solubility. We used relatively low levels of trichothecenes and used the protocol recommended by the manufacturer with regard to extraction, i.e., we used phosphate buffer saline as an aqueous solvent. However, to exhaustively extract plant tissues especially if high levels of trichothecenes are present, it might be more appropriate to use a more non-polar extraction solvent, followed by evaporation and resuspension into buffered saline. All of these factors can influence the overall detection limits of chemical moieties.

Overall, the ELISA method is both rapid and sensitive. However, because of cross-reactivity of this ELISA protocol, methods such as HPLC will be required to separate and quantify specific trichothecenes. To our knowledge, this is the first work using ELISA to study trichothecene absorption and translocation in tissues of young kudzu seedlings and the absorption of trichothecenes by hemp sesbania seedlings. However, because of the lack of specificity of this ELISA method and the possible positive reaction of similar molecular structures with the antibodies, we are presently uncertain if any molecular transformation, degradation, or metabolism of the applied trichothecene molecules occurred within the plant tissues. This metabolic aspect and a more complete trichothecene production profile of MV is currently being pursued using HPLC methodology as we acquire high-purity standards of trichothecene analogs and their metabolites. Hence combining ELISA and HPLC protocols in our research program probably offers the most practical approach toward attaining our overall goals for this project as outlined elsewhere (Hoagland, Boyette, and Weaver 2007; Hoagland, Weaver, and Boyette 2007).

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