Phytotoxicity and mammalian cytotoxicity of macrocyclic trichothecene mycotoxins from *Myrothecium verrucaria*

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Abstract

Macrocyclic trichothecene toxins produced by *Myrothecium verrucaria* (a phytopathogen of interest in biological weed control) and the non-trichothecene toxin atranone B from *Stachybotiys atra* were tested for phytotoxicity in duckweed (*Lemna pausicostata* L.) plantlet cultures and kudzu (*Pueraria lobata* L.) leaf disc assays, and for mammalian cytotoxicity in four cultured cell lines. Roridin E and H, epi-isororidin E, and verrucarin A and J were phytotoxic (half-maximal effect in the concentration range 0.1–9.7 μM on duckweed and 1.5–>80 μM on kudzu) and cytotoxic to mammalian cell lines (half-maximal inhibition of proliferation in the concentration range 1–35 nM). Trichoverrins A and B and atranone B were moderately phytotoxic (half-maximal effect in the concentration range 1 9–69 μM on duckweed and 13–>80 μM on kudzu) and weakly cytotoxic with mammalian cell lines (half-maximal inhibition of proliferation in the concentration range 0.3–>2 μM). © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Kudzu, *Pueraria montana Leguminosae,* Macrocyclic trichothecenes; Mycotoxins; Natural products; Mycoherbicide; *Mvrothecium verrucaria*; Phytotoxins; Phytotoxicity; Cytotoxicity; Biological control

1. Introduction

Myrothecium verrucaria has been investigated as a biological control agent against kudzu (*Pueraria lobata* L.) (Boyette et al., 1999; 2002), an invasive exotic weed that causes major economic losses in fields, roadsides and forested areas of southern United States (Miller, 1977; Mitich, 2000). Isolates of *M. verrucaria* also attack other weed species (Walker and Tilly, 1997; Yang and Jong, 1995a,b). In culture a phytopathogenic isolate of *M. verrucaria* produced a series of macrocyclic trichothecenes (Fig. 1), which were identified as epiroridin E, epi-isororidin E, roridin E, roridin H, trichoverrin A, trichoverrin B, verrucarin A and verrucarin J (Jarvis et al., 1982, 1985). However, none of these compounds could be detected in extracts of kudzu, soybean (*Glycine max*), and sicklepod (*Cassia obtusifoloa*) plants at 1, 2, 3, 4, 5, 7, 10 and 14 days after spraying with *M. verrucaria* spore preparations (Abbas et al., 2001), nor could

viable *M. verrucaria* be detected. Nevertheless, it has been suspected that these toxins may play a role in the phytopathogenicity of *M. verrucaria* and related fungal species (Bean et al., 1984, 1988; Cunfur et al., 1969; Cunfer and Lukezic, 1970; Cutler, 1988; Kuti et al., 1987, 1989). Macrocyclic trichothecenes are believed to act by inhibition of protein synthesis by inhibiting the peptide bond formation step (McLaughlin et al., 1977). The presence, persistence and toxicity of macrocyclic trichothecenes produced by *M. verrucaria* must be evaluated (Grove, 1993; Jarvis, 1991; Mortimer et al., 1971; Ueno, 1983) before spore preparations or fermentation products can be further considered as possible biological control agents for kudzu. Macrocyclic trichothecenes are highly toxic; extreme care should be exercised when handling pure toxins, as well as *M. verrucaria* mycelium, spores or suspensions for field application.

In the present study, two in vitro bioassay systems for phytotoxicity and one for mammalian toxicity were selected to provide an initial evaluation of efficacy and safety as potential weed control agents. The three test systems were used to individually evaluate each of the major macrocyclic trichothecenes (Fig. 1) produced by *M. verrucaria,*

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with the exception of epiroridin E, which was not available as a pure compound. Atranone B (Fig. 1), a metabolite isolated from the toxigenic mold *Stachybotrys atra,* was also included, because it has been found in conjunction with macrocyclic trichothecenes (Hinkley et al., 1999). Preliminary observations on the phytotoxicity of macrocyclic trichothecenes have been made by (Cutler and Jarvis, 1985), who tested roridin A, isororidin E, verrucarin A and J and trichoverrin B on wheat, bean, corn and tobacco. All inhibited etiolated wheat coleoptile growth, and caused chlorosis and stunting in intact plants (Cutler and Jarvis, 1985). The phytotoxicity of macrocyclic trichothecenes has also been tested on *Baccharis* cell lines (Jarvis et al., 1988) and in muskmelon tissues (Kuti et al., 1987).

2. Results and discussion

Roridin and verrucarin derivatives were highly phytotoxic to duckweed, causing increased cellular leakage, growth inhibition and chlorophyll loss with increasing concentration to 10 μM. Verrucarin A was the most toxic. At 0.5 μM it caused a 422 μmho/cm increase in cellular leakage, 48% reduction in chlorophyll and 44% growth inhibition after 72 h exposure. Roridin H was the least phytotoxic. At 0.5 μM, roridin H caused a 19 μmho/cm increase in cellular leakage, 6% chlorophyll loss and no growth inhibition in duckweed cultures. Higher concentrations of these toxins were required to obtain toxic effects in the kudzu leaf disc assay system. Table 1 shows the toxin concentrations needed to cause half-maximal toxic effects, according to selected criteria representing strongly toxic effects. By these criteria, the corresponding values for half-maximal toxicity of trichoverrin A and B and atranone B were all >80 μM. However, these toxins induced weaker, but, nevertheless, still substantial toxic effects for the concentrations tested. Table 2 shows the toxin concentrations needed to cause half-maximal toxic effects, according to selected criteria representing weakly toxic effects. The most active compound was trichoverrin B, which at 20 μM caused an 87 μmho/cm increase in cellular leakage, 6% chlorophyll loss and 16% growth inhibition in duckweed cultures. Atranone B at 20 μM induced the least phytotoxic effects, including a 28 μmho/cm increase in cellular leakage, 3% chlorophyll reduction, and 17% growth inhibition in duckweed cultures. The phytotoxic effects increased up to the highest concentration tested (80 μM). In the kudzu leaf disc assay, a parallel pattern of toxicities was seen, but at higher concentrations.

Verrucarin A and J were very cytotoxic to the four mammalian cell lines (Table 1). The approximate half-maximal inhibition concentrations (IC_{50}) were 0.5–3.5 μM. Roridin derivatives were cytotoxic as well, and IC_{50} values ranged from 1.5 to 20 μM. Trichoverrin A and B
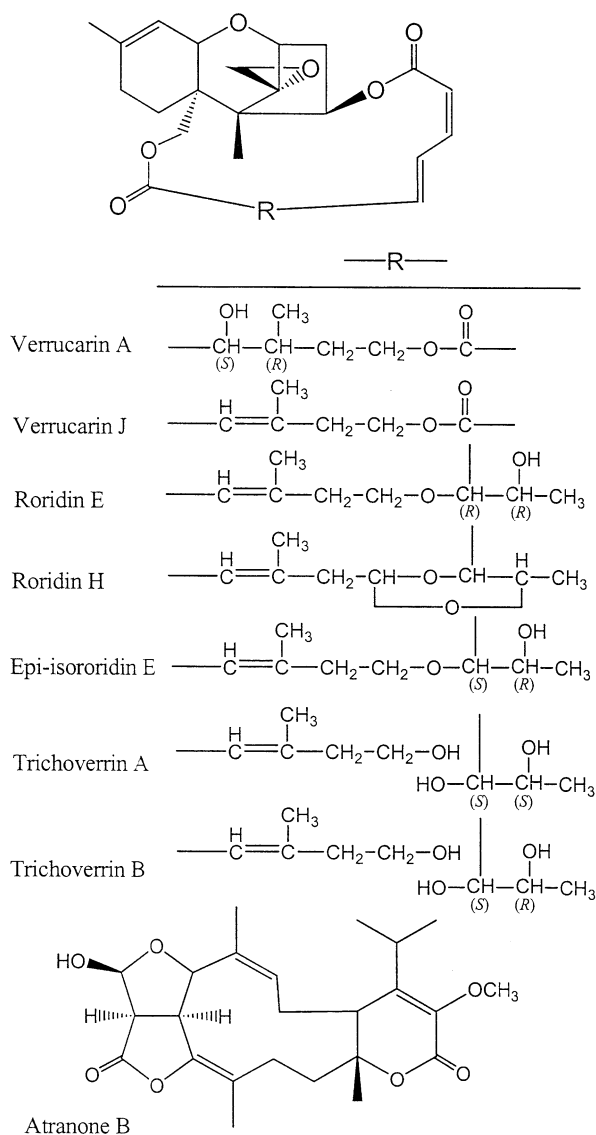

Fig. 1. Structures of mycotoxins included in this study.

and atranone B produced weak cytotoxicity (IC_{50} values >100 μM) in four mammalian cell lines (Table 2).

M. verrucaria is an effective pathogen of kudzu, but the pathogenic mechanism is unknown. Macrocyclic trichothecenes tested individually are clearly phytotoxic to duckweed and kudzu. Although they are produced in large quantities by *M. verrucaria* in culture, macrocyclic trichothecenes could not be found in extracts of treated plants (Abbas et al., 2001). They could, nevertheless, play a role in pathogenesis (Bean et al., 1984), if they were present in amounts too small to be detected, or if they were metabolized by the plants to other bioactive congeners.

Some differential toxicity was observed between atranone B and trichoverrins A and B, which retained moderate phytotoxicity, but exhibited much reduced mammalian cytotoxicity relative to the other macrocyclic trichothecenes tested. The toxic mechanisms on

Table 1

Toxic responses to potent macrocyclic trichothecene mycotoxins from *Myrothecium verrucaria*[a]

| Toxicity test | Trichothecene concentration causing the indicated toxic respnoses ±s.e. | | | | |
	Verrucarin A	Verrucarin J	Roridin E	Roridin H	Epi-isororidin E
Phytotoxic responses in cultures of duckweed plantlets after 72 h exposure (μM)					
(a) Electrolyte release into the medium ($EC_{400\mu mho/cm}$)	0.39±0.06	2.4±0.6	2.8±0.6	5.7±1.0	4.3±1.8
(b) Chlorophyll reduction in plantlets ($EC_{25\%}$)	0.11±0.00	1.5±0.6	1.3±0.2	2.3±0.5	1.8±0.9
(c) Growth inhibition as wet weight reduction ($IC_{50\%}$)	0.85±0.03	5.2±1.9	3.7±0.3	9.7±0.0	6.2±2.3
Phytotoxic responses in kudzu leaf disc cultures after 48 h exposure (μM)					
(a) Electrolyte release into the medium ($EC_{100\mu mho/cm}$)	7.3±0.0	1.5±0.1	8.4±1.7	13±0.0	5.1±0.4
(b) Chlorophyll reduction in leaf discs ($EC_{25\%}$)	79±2	50±0	26±3	>80	17±6
Mammalian cytotoxicity measured as inhibition of proliferation by cultured cell lines (IC_{50}) (nM)					
(a) H4TG	2.50±0.00	4.56±0.70	3.45±0.42	10.8±0.0	34.8±2.2
(b) MDCK	0.96±0.14	2.02±0.14	1.74±0.62	3.98±0.56	7.32±1.09
(c) NIH3T3	3.03±0.0	4.55±0.30	2.30±0.46	9.84±0.38	34.4±3.9
(d) KA31T	1.94±0.0	3.14±0.04	7.68±1.96	8.64±0.70	21.3±3.0

[a] Abbreviations: EC = effective concentration; IC = inhibitory concentration; s.e. = standard error of the estimate.

Table 2

Toxic responses to weak mycotoxins from *Myrothecium verrucaria*[a]

| Toxicity test | Trichothecene concentration causing the indicated toxic responses ± s.e. (μM) | | |
	Trichoverrin A	Trichoverrin B	Atranone B
Phytotoxic responses in cultures of duckweed plantlets after 72 h exposure			
(a) Electrolyte release into the medium ($EC_{150\mu mho/cm}$)	38±15	19±5	40±14
(b) Chlorophyll reduction in plantlets ($EC_{25\%}$)	51±18	54±4	69±0
(c) Growth inhibition as wet weight reduction ($IC_{25\%}$)	58±13	30±2	56±4
Phytotoxic responses in kudzu leaf disc cultures after 48 h exposure			
(a) Electrolyte release into the medium ($EC_{50\mu mho/cm}$)	44±0	69±4	13±1
(b) Chlorophyll reduction in leaf discs ($EC_{25\%}$)	>80	>80	>80
Mammalian cytotoxicity measured as inhibition of proliferation by cultured cell lines (IC_{50})			
(a) H4TG	>2.0	>2.0	22.2±4.0
(b) MDCK	0.28±0.04	0.32±0.08	12.7±2.8
(c) NIH3T3	0.59±0.01	0.50±0.07	29.1±2.9
(d) KA31T	0.70±0.13	0.63±0.15	18.2±2.3

[a] Abbreviations: EC = effective concentration; IC = inhibitory concentration; s.e. = standard error of the estimate.

these toxins is poorly understood, particularly in plants. This suggests that these toxins may serve as initial leads for the future development of analogs with more potential for use in weed control, specifically of kudzu. Success in this endeavor would require identification of derivatives with simpler structures and enhanced differential toxicity. Individual *M. verrucaria* toxins should be considered as possible biological control agents for kudzu only if they exhibit substantial phytotoxicity coupled with low mammalian cytotoxicity.

3. Experimental

3.1. Source of phytotoxins

The seven macrocyclic trichothecenes (Fig. 1) used in these studies (epi-isororidin E, roridin E, roridin H,

trichoverrin A, trichoverrin B, verrucarin A, and verrucarin J) were isolated and characterized as reported (Jarvis et al., 1982). Atranone B (Fig. 1) was isolated and purified as reported (Hinkley et al., 1999). The purity of all toxins was established by thin layer chromatography. All toxins were dissolved in 0.5% DMSO in sterile distilled water to produce a 1 mM stock solution; dilutions were made in the culture medium used in each assay at 0, 0.5, 1, 5, 10, 20, 40 and 80 μM. The appropriate test concentrations were selected on the basis of a preliminary toxicity test.

3.2. Phytotoxicity bioassays in duckeed

Cultures of duckweed (*L. pausicostata* Helgelm. 6746) were initiated and grown as described previously (Tanaka et al., 1993). Bioassays were performed as previously described (Abbas et al., 1998a, b). Briefly, 10 colonies of 3

fronds each were transferred to 3-cm polystyrene Petri dishes containing test toxins in two concentration ranges: 0, 0.5, 1, 5, and 10 μM for roridin and verrucarin derivatives; and 0, 0.5, 1, 5, 10, 20, 40, and 80 μM for trichoverrin A and B and atranone B. Plants were then covered with three layers of cheesecloth and incubated at 25 °C under continuous light for up to 72 h at 500 μE $m^2 s^{-1}$ (125 μE $m^2 s^{-1}$ under the cheesecloth) in a growth chamber. The plants were examined for visual signs of phytotoxicity, and the conductivity of the bathing medium was monitored at each of 0, 24, 48 and 72 h. Two electrolyte leakage experiments were conducted in triplicate. Reported results are means ±standard error of triplicate samples from representative experiments. Bleaching was measured at 72 h by DMSO extraction of total chlorophyll and spectrophotometric quantitation (Hiscox and Israelsta, 1979). Growth inhibition was measured as reduced gain in fresh weight relative to controls. Half-maximal toxic concentrations were selected by interpolation from a least squares line fitted to data plotted as response versus log of toxin concentration.

3.3. Phytotoxicity bioassays in kudzu

Kudzu *(Pueraria lobata)* seeds were purchased from Adams-Briscoe Seed Co., Jackson, GA 30233, USA. Plants were grown to the 6- to 7-leaf stage and the 4th and 5th mature leaves were used. The plants were greenhouse grown in 4-in pots containing a 1:1 commercial potting mix (Jiffy Mix®): soil combination supplemented with a controlled- release 13:13:13 (N:P:K) fertilizer, at 28–32 °C with 40–60% relative humidity. The photoperiod was approximately 14 h, with 1600–1800 μ mol m ^{2} s^{-1} photosynthetically active radiation (PAR) at midday, as measured with a light meter for 14 days. This assay was carried out essentially as described previously (Abbas et al., 1993; 1995). Briefly, twenty 4-mm diameter kudzu leaf discs were cut with a cork borer from healthy leaves, washed in 1% sucrose in 1 ml of MES (2-[*N*-morpholino]ethanesulfonic acid) (pH 6.5), and then placed in 3-cm diameter polystyrene Petri dishes with 3 ml wash medium with, or without toxin. Incubations and phytotoxicity assessments were carried out under the same conditions as for the duckweed bioassay, except that concentrations of 10, 20, 40 and 80 μM were used for all compounds for a maximum incubation period of 48 h, and no evaluation of growth was conducted.

3.4. Mammalian cytotoxicity bioassay

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. Other cell lines 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., 1984; Shier, 1991; Shier et al., 1991). Briefly, each cell line was cultured in 96-well trays at 10^4 cells/well in triplicate in 0.2 ml of 5% (vol/vol) calf serum (HyClone Laboratories, Logan, UT, USA) in Dulbecco's modified Eagle's medium containing three independent serial dilutions (two 2-fold dilutions followed by a 2.5-fold dilution) of each toxin. The cultures were incubated under normal culture conditions (Shier, 1991) for fixed time periods from 3–5 days. The time period selected was that required for control wells with no toxin to become confluent (KA31T, 3 days; MDCK and NIH3T3, 4 days; H4TG, 5 days). The cells were fixed with 10% formalin in saline and stained with 0.05% (w/v) crystal violet in 20% (v/v) aqueous methanol. Cell number was estimated by counting at least 250 cells in 5 microscope fields in 3 wells. Thus, the criteria for viability were attachment and proliferation. The concentration causing half-maximal inhibition of cell proliferation (IC_{50}) was estimated graphically by interpolation using straight lines fitted by the least squares method to plots of cell number versus log concentration.

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