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# Isolation and Partial Characterization of Phytotoxins Produced by *Exserohilum monoceras*, a Potential Bioherbicide for Control of *Echinochloa* Species

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**Abstract**

*Exserohilum monoceras* (Drechsler) Leonard and Suggs causes leaf blight in *Echinochloa* species and is presently being evaluated as a potential bioherbicide for the control of *Echinochloa* species. Bioassays had shown that *E. monoceras* produces phytotoxins biologically active against *Echinochloa* species. Two biologically active compounds, designated Toxin I and Toxin II were isolated from *E. monoceras* culture filtrate and inoculated plant leaves by means of extraction and thin layer chromatography (TLC). On TLC plates, $R_f$ values of Toxin I and Toxin II were 0.59 and 0.72, respectively. In the absence of the pathogen, each of the purified toxins incited symptoms similar to those caused by *E. monoceras* conidia. Toxin I was shown to be most potent and host-specific. Toxin II had a broader spectrum of activity, but played a less important role in producing disease on *Echinochloa* species. A root inhibition experiment indicated that Toxin I was selective for *Echinochloa* species and did not damage rice.

**Keywords:** bioherbicide, biological control, *Echinochloa, Exserohilum monoceras*, phytotoxin.

*Exserohilum monoceras* (Drechsler) Leonard and Suggs causes leaf blight in *Echinochloa* species and is presently being evaluated as a potential bioherbicide for the control of *Echinochloa* species (Zhang *et al.*, 1996; Zhang and Watson, 1997). Inoculation with *E. monoceras* resulted in a blight-like reaction characterized by chlorosis as well as a diffuse, water-soaking reaction appearing two days after inoculation. This was followed by a rapid necrosis of affected tissue, but often, with an absence or only a weak expression of typical lesions. The symptoms incited by *E. monoceras* indicated that phytotoxins might be operative in pathogenesis. This assumption was supported by histological investigations of *Echinochloa* leaves inoculated with *E. monoceras*. After hyphal penetration, chlorosis and water-soaked lesions developed rapidly in advance of the hyphae, suggesting the presence of diffusible substances. Furthermore, intact seedling bioassays demonstrated that *E. monoceras* produces phytotoxins that were biologically active against *Echinochloa* species (Zhang *et al.*, 1996).

The first chemical substance isolated from *E. monoceras* culture was monocerin. This

substance was not characterized as a phytotoxin but was described as an antibiotic to protect wheat (*Triticum aestivum* L.) against powdery mildew (*Erysiphe graminis* D.C. ex M rat) (Robeson and Strobel, 1982). Subsequently, monocerin has also been isolated from *Exserohilum turcicum* (Pass.) Leonard et Suggisit and found to have phytotoxic activity on johnsongrass (*Sorghum halepense* (L.) Pers.) and Canada thistle (*Cirsium arvense* (L.) Scop.) (Robeson and Strobel, 1982). However, there have been no reports of phytotoxin production by *E. monoceras*.

The present study was initiated to develop methods for production, detection, isolation, and partial characterization of phytotoxins produced by the fungal pathogen *E. monoceras*.

## Materials and methods

**Culturing.** The *E. monoceras* culture used in this study was originally isolated from naturally infected *Echinochloa* species leaves collected in the Philippines. The organism was maintained on half-strength potato dextrose agar (1/2 PDA; Difco, Detroit, MI) slants in small vials under mineral oil at 4°C (Zhang *et al.*, 1996). For toxin production, the fungus was grown in l-L Roux bottles containing 200 ml of Modified Fries medium (100 g sucrose, 2 g casein hydrolysate, 1.5 g $NaNO_3$, 1 g $K_2HPO_4$, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, and distilled water to 1 L) (Tuite, 1969). Cultures were incubated at laboratory temperature (25 ± 2 °C) on a rotary shaker operating at 150 rpm.

**Isolation and purification of toxins.** After 21 days of growth, the culture fluid was obtained by filtrating through three layers of cheesecloth and concentrating culture filtrates to 10% of their original volume by using a flash evaporator at 50°C (Steiner and Strobel, 1971; Stierle *et al.*, 1992). The concentrated broth was extracted with chloroform ($CHCl_3$) (3 x 1/3 vol). The chloroform extract was then evaporated using a flash evaporator, the residue was weighed, and collected in vials using chloroform. The chloroform extract was subjected to Thin Layer Chromatography (TLC). Analytical silica gel plates, 0.25 mm thick, were used and developed in chloroform: methanol 9:1. Bands on TLC plates were marked under ultra violet (UV) light and then each band was carefully cut off. Compounds from each band (with silica gel) were re-extracted in chloroform, dried by flash evaporator, and weighed. In order to detect biological activity, each component was prepared and subjected to a leaf bioassay by placing in 2% aqueous ethanol solution containing 0.05% Tween 20 as a wetting agent.

**Leaf bioassay.** The most recently expanded leaf of each of three *Echinochloa* species (i.e. *E. crus-galli*, *E. colona*, and *E. glabrescens*) was detached from plants. Sets of glass slides were prepared by inserting both ends of the slide into filter paper (Whatman No. 3) so that a 4 cm mid-portion was exposed. The tip and basal portion of a detached leaf were fixed by inserting them between the slide and filter paper and by placing a small cotton ball on the basal end of the detached leaf (to keep the leaf from drying). The prepared leaf set was placed into a petri dish. The filter paper and cotton ball were then moistened with distilled water. The detached leaf was wounded with a glass capillary tube and a droplet of test solution containing 50 µg/ml of the toxin in 2% aqueous ethanol with 0.05% Tween 20 was placed on the wound (Karr *et al.*, 1974). The plates were incubated at 28°C in the dark. After 48 h incubation, symptoms similar to those produced by conidia were observed.

**Isolation of toxins produced in vivo.** *E. crus-galli* seedlings at the 4-leaf stage were inoculated with *E. monoceras* at a rate of 1 x $10^8$ conidia/$m^2$, placed in a dew chamber for

24 h, and then transferred to a mist room (Yeh and Bonman, 1986). The control treatment consisted of plants that were inoculated with distilled water but otherwise were subjected to the same conditions as inoculated plants. After 1 week, 30 g of severely infected leaf material was collected, chopped, and treated overnight with 350 ml of chloroform and methanol at room temperature (Vidhyasekaran *et al.*, 1986). Extracts were filtered through four layers of cheesecloth. Residues of methanol were added to 350 ml of chloroform for further extraction for 4 h and once again filtered through four layers of cheesecloth as well. All the chloroform, methanol, methanol + chloroform filtrates were further filtered through two layers of Whatman No. 1 filter paper and 100 ml of water was added to them. Solvents were removed by using a flash evaporator and water fractions were partitioned with chloroform (3 x 1/3 vol). The water fraction was discarded and the chloroform was evaporated to dryness in vacuum. Residues were then collected in small vials and then subjected to TLC. Analytical plates, 0.25 cm thick, were run in chloroform: methanol 9:1.

**Host specificity of toxins.** Nine plant species including three *Echinochloa* species and cultivars of three types of rice were selected for host specificity testing, using leaf bioassays. Leaf sections of test species were inoculated with *E. monoceras* by preparing a spore suspension containing approximately 5 x $10^7$ conidia/ml in 2% aqueous ethanol with 0.05% Tween 20 and placing 50 ml of the suspension on a leaf wound as described above. The toxins were also diluted to 50 µg/ml in 2% aqueous ethanol containing 0.05% Tween 20 and tested on leaves of these same hosts.

**Root growth inhibition.** A single batch of seeds of each of the three *Echinochloa* species, *E. crus-galli*, *E. colona*, and *E. glabrescens* collected from natural agricultural *Echinochloa* populations on the International Rice Research Institute (IRRI) farm was used in this experiment. The rice cultivars used were Dee-Geo-Woo-Gen and Chianan, representing the indica and japonica rice types, respectively. Seed of each *Echinochloa* species and rice cultivars was incubated in petri dishes on moistened filter paper at room temperature (25 ± 2°C) for 48 h. Seedlings having primary roots 5 mm long were selected and placed in 5 cm diameter petri dishes (5 germinated seeds/dish). Dishes contained 2 ml of the toxin preparation diluted with 2% aqueous ethanol solution at concentrations of 0, 0.2, 0.4, 2, 4, 20, 40, 60, 80, or 100 µg/ml. After 48 h at room temperature (25 ± 2°C), the root length of seedlings was measured. Percent root growth inhibition was obtained by comparing the root length of seedlings in the presence of toxins to that of controls (Yoder *et al.*, 1977). There were 25 measurements of seedling root length for each treatment or control.

Comparison of toxins with standards of bipolaroxin and exserohilone. Dr. G.A. Strobel provided the standards of two phytotoxins, bipolaroxin and exserohilone. These two standards and Toxins I and II were subjected to TLC. Analytical silica gel plte, 0.25 mm thick were used and developed in chloroform:methanol 9:1. The $R_f$ value was recorded for each compound. Compounds with the same $R_f$ value were considered to be the same compound (Stierle *et al.*, 1992).

## Results

**Isolation and purification of toxins.** No toxin activity was detected in the water fraction after partitioning with three volumes of chloroform. However, toxin activity was detected in the chloroform fraction. The chloroform extract contained six different compounds, including the two toxin fractions, detected as short UV-quenching bands. The two toxin fractions migrated with $R_f$ values of 0.59 (toxin I) and 0.72 (toxin II) (chloro-

form:methanol = 9:1 solvent) on the TLC plate. When these two bands were eluted separately and rechromatographed, no other spots were detected. In different solvent systems, the toxin fractions migrated with different $R_f$ values. In chloroform:methanol 25:1, toxin I and toxin II migrated as short UV-quenching bands, with $R_f$ values of 0.42 and 0.65, respectively. In pentane:ethyl ether: acetic acid 20:80:1, toxin I and toxin II migrated at $R_f$ values of 0.48 and 0.29, respectively.

Visually, toxin I appears as a yellow powder and toxin II as an orange powder. They are highly soluble in chloroform, methanol, ethanol and acetone, and sparingly soluble in water. Both toxins are fairly stable to heat. Neither autoclaving at 121 °C for 15 min, nor storing at room temperature, changed the $R_f$ value in the chloroform:methanol system while retaining full toxicity to *Echinochloa*.

**Phytotoxicity of toxins.** Toxin I appears to be more potent than toxin II. In the leaf bioassay using toxin I, first symptoms appeared within 24 h as a weak chlorotic marbling which subsequently developed into well-defined chlorotic spots surrounding brown necrotic lesions. Symptoms on detached *Echinochloa* leaves produced by toxin I were similar to those produced by the conidia of the pathogen after 48 h. Toxin II qualitatively induces the same symptoms as toxin I. However, the minimum toxin concentration required to cause chlorosis was 7 to 8 times greater than that of toxin I.

**Specificity of toxins.** *E. monoceras* severely infected *Echinochloa* species and slightly infected corn (*Zea mays* L.), but did not infect other hosts tested (Table 1). Toxin I induced typical symptoms on leaves of plants susceptible to the fungus but did not produce any effects on non-hosts. Symptom expression on *Echinochloa* species with toxin II was much weaker and toxin II had a broader spectrum of activity, including grass and broad-leaved plant species (Table 1).

**Table 1.**
**Host range of Exserohilum monoceras and its associated toxinsa**

| Plant species | Pathogenicity[b] | | |
|---|---|---|---|
| | Conidia | Toxin I | Toxin II |
| *Echinochloa crus-galli* | +++ | +++ | + |
| *Echinochloa colona* | +++ | +++ | + |
| *Echinochloa glabrescens* | +++ | +++ | + |
| Rice (*Oryza sativa* L.) | | | |
| Indica type | - | - | - |
| Japonica type | - | - | - |
| Tropical Japonica type | - | - | - |
| Corn (*Zea mays* L.) | + | ++ | - |
| Tomato (*Lycopersicon esculentum* L.) | - | - | ++ |
| Banana (*Musa sapientum* L.) | - | - | + |
| Mungbean (*Vigna radiata* (L.) R. Wilcz.) | - | - | + |
| Cowpea (*Vigna unguiculata* (L.) Walp.) | - | - | + |

[a] Toxin I with $R_f$ 0.59 and toxin II with $R_f$ 0.72.

[b] Symbols: +++ = severe, ++ = moderate, and - = no symptoms.

**Root growth inhibition.** Root growth of *Echinochloa* species was much more susceptible to toxin I than was rice (Fig. 1). Concentrations of 0.2 µg/ml of toxin I inhibited *Echinochloa* spp. root growth by 46-60%, but did not inhibit rice root growth. Similarly, the 20 µg/ml toxin I treatment inhibited *Echinochloa* spp. root growth by approximately 80%, whereas rice root growth was inhibited by less than 10% by this treatment. The root growth suppression response to toxin I by the three *Echinochloa* species was similar.

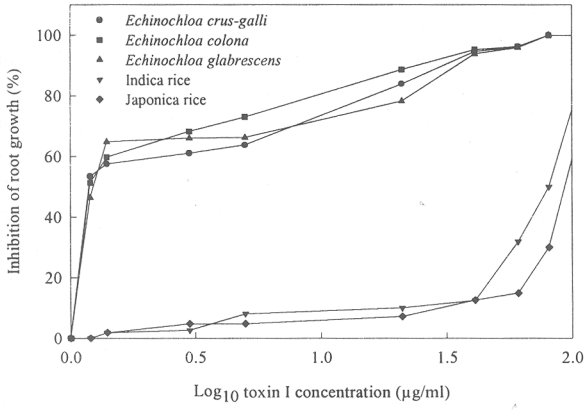


**Figure 1.** Inhibition of seedling root growth of rice and Echinochloa species by toxin I produced by Exserohilum monoceras.

## Discussion

Toxins I and II are not bipolaroxin or exserohilone based on comparison with the standards provided by Dr. G.A. Strobel. However, toxin II is most likely monocerin since it has the same $R_f$ value when run in the same solvent system and similar conditions reported elsewhere (Robeson and Strobel, 1982). If so, monocerin also has phytotoxic properties towards *Echinochloa* species besides those reported for Canada thistle, johnsongrass, tomato, and cucumber (Robeson and Strobel, 1982). Toxin I might be a novel phytotoxin that is highly active on *Echinochloa* species. However, further research is required to be able to properly identify these toxins.

Pringle and Scheffer (1964) defined a host-specific toxin as a metabolic product of a pathogen that is toxic only to the host of the pathogen. Toxin I produced by *E. monoceras* has several characteristics in common with other host-specific toxins (Steiner and Byther, 1971). The host range of Toxin I was similar to that of the pathogen. Toxin II, if it is monocerin, is known to have a very broad spectrum of biological activity including antibiotic, insecticidal, and phytotoxic properties (Robeson and Strobel, 1982).

These two toxins were also isolated from *Echinochloa* leaves inoculated with *E. monoceras*. Therefore, it can be concluded that the two toxins isolated from cultures are also produced during the infection of *Echinochloa* species by *E. monoceras*. It appears that toxin I contributes more than toxin II to disease expression in *Echinochloa* species because toxin I is more potent than toxin II.

There has been considerable research interest in phytotoxins produced by plant pathogens of crop plants. In several instances, these phytotoxins have proven useful as tools for screening plants for toxin insensitivity (resistance) and as probes of normal physiological plant function (Strobel, 1982). Phytotoxins produced by weed pathogens have received less attention. However, phytotoxins produced by weed pathogens have the potential to be used directly on the target weed species or utilized as building blocks for novel herbicides (Duke, 1986; Hoagland, 1990; Strobel *et al.*, 1992). The selectivity of

toxin I toward rice and *Echinochloa* plants indicate that toxin I has the potential to be used as one of these novel herbicides or building blocks.

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