Plant Pathol. J. 20(1) : 52-57 (2004)

Isolation and Partial Characterization of Phytotoxic Mycotoxins Produced by *Sclerotinia* sp., a Potential Bioherbicide for the Control of White Clover (*Trifoliorum repens*)

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(Received on December 5, 2003; Accepted on January 20, 2004)

***Sclerotinia* sp. (isolate BWC98-105) causes stem blight and root rot in *Leghum* sp., and is presently being evaluated as a potential mycoherbicide for the control of *Trifoliorium repens*. Bioassays have shown that *Sclerotinia* sp. produces phytotoxic substance which is biologically active against *T. repens*. Two biologically active compounds, designated as compounds I and II, were produced *in vitro* from the culture filtrate of BWC98-105 isolate *Sclerotium* sp. Compounds I and II were purified by means of liquid-liquid extraction and C_{18} open column chromatography (300 × 30 mm, i.d). To determine the purity, the purified compounds were analyzed by RP-HPLC. The analytical RP-HPLC column was a TOSOH ODS-120T (150 × 4.6 mm i.d, Japan), of which the flow rate was set at 0.7 mL/min using the linear gradient solvent system initiated with 15% methanol to 85% methanol for 50 min with monitoring at 254 nm. Under these RP-HPLC conditions, compounds I and II eluted at 3.49 and 4.13 min, respectively. Compound II was found to be most potent and host specific. However, compound I had a unique antibiotic activity against phytopathogenic bacteria like bacterial leaf blight (*Xanthomonas oryzae*) on rice, where it played a less important role in producing toxicity on *T. repens*. No toxin activity was detected in the water fraction after partitioning with several organic solvents. However, toxin activity was detected in the ethyl acetate and butanol fractions. In the leaf bioassay using compound II, the disease first appeared within 4-5 h as water soaked rot, which subsequently developed into well-defined blight affecting the whole plant.**

Keywords **:** *Trifoliorum repens,* mycoherbicide, phytotoxic mycotoxin, *Sclerotinia* sp. BW98-105

Sclerotinia sp. isolate BWC98-105 causes leaf, stem, and root blight in *Leghum* species, and is presently being evaluated as a potential mycoherbicide for the control of *Leghum* sp. weeds (Hong et al., 1998, 1999, 2001, 2002). White root rot of white clover (*Trifoliorum repens*) caused by isolate BWC98-105 was reported for the first time in Korea. Typical symptom on the root included formation of water soaked brown rot, resulting in complete blight of the top parts of the plant. This was followed by rapid necrosis of affected tissue, but often, with an absence or only a weak expression of typical lesions. The symptoms incited by the fungus indicated that pathotoxin might be operative in pathogenesis. 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 the fungus produces phytotoxic mycotoxins that were biologically active against *Leghum* sp. (Hong et al., 1999, 2001).

Chemical substance extracted from the fungus culture was phytotoxic mycotoxin, in which monocerin has been isolated from *Exserohilum turcicum* (Pass.) Leonard and found to have phytotoxic activity on johnson-grass (*Sorghum halepense* (L.) Pets.) and Canada thristle (*Cirsium arvense* (L.) Scop.) (Robeson and Strobel, 1982). However, there have been no reports of herbicidal phytotoxic mycotoxin production by *Sclerotinia* sp. The present study was initiated to develop methods for production, detection, isolation, and partial characterization of phytotoxic mycotoxins produced by the mycoherbicide isolate BWC98-105.

Materials and Methods

Fungal culture. The *Sclerotinia* sp. culture used in this study was originally isolated from naturally infected *T. repens* root which was collected from the field of the National Yeongnam Agricultural Experiment Station (NYAES), Milyang, Korea. 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 250 Erlenmeyer flask containing 200 ml of Czapek Dox

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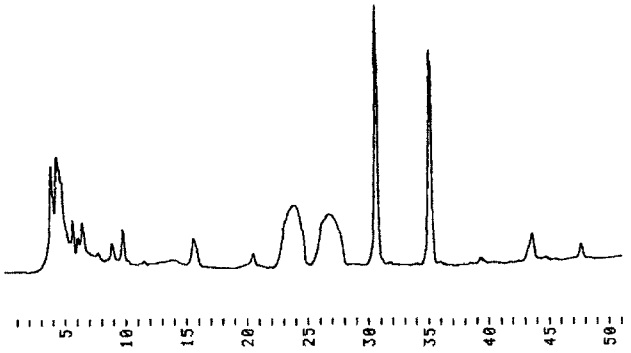

Fig. 1. HPLC chromatogram of extract from culture solution of BWC98-105 isolate *Sclerotinia* sp.

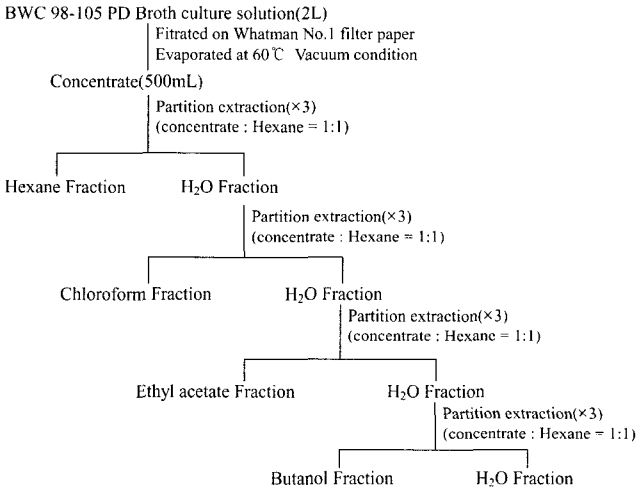

Fig. 2. Procedure of liquid-liquid partition extraction for culture solution of BWC98-105 isolate *Sclerotinia* sp.

broth medium (24 g medium and distilled water to 1 L; Difco, Detroit, MI). Cultures were incubated at temperatures of 28 ± 1°C on a rotary shaker operating at 150 rpm for 7 days.

Isolation and purification of toxins. After 7 days of growth, the culture fluid was obtained by filtrating through three layers of cheesecloth, and centrifuged at 8,000 rpm for 20 min. Culture filtrates were concentrated to 10% of their original volume by using a flash evaporator at 60°C (Steiner et al., 1971; Stierle et al., 1992). The initial separation of active substances from the culture filtrate follows the solvent fractioning procedure shown in Fig. 1. Dual solvent systems used n-hexane, chloroform, ethyl acetate, n-butanol, and water as linear separation system (Fig. 2). Briefly, the same amount of each solvent was equaled and mixed by shaking for 3 min in a separation funnel with 150 ml of the culture filtrate or aqueous layer, and separation was made between the solvent and the aqueous layers. Each extract was then evaporated using a flash evaporator, and the residue was weighed and collected in vials using methanol. 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. Compounds I and II were purified by means of liquid-liquid extraction and C_{18} open column chromatography (300 × 30 mm, i.d) (Fig. 4). To determine the purity, the purified toxin I and toxin II were analyzed by RP-HPLC (Hitachi, Japan). The analytical RP-HPLC column was a TOSOH ODS-120T (150 × 4.6 mm i.d, Japan), and the flow rate was set at 0.7 mL/min by using a linear gradient solvent system initiated with 15% methanol to 85% methanol for 50 min with monitoring at 254 nm under these RP-HPLC conditions.

Leaf bioassay. Fully emerged leaves of *T. repens* were tested. The basal portion of the leaves were covered by bending them with cotton (10 × 10 × 0.5 mm) soaked with each concentrated component containing 1.5 μg/ml of the toxin in 2% aqueous ethanol with 0.05% Tween 20, and then by placing them in a dew chamber for 24 h to keep the leaves from drying. Leaf sample moistened with 2% aqueous ethanol with cotton was used as control. The pots were incubated at 25°C in the dark dew chamber. After 24 h incubation, symptoms similar to those produced by mycelia were observed.

Host specificity of the toxins. Twelve plant species including four lawn grass species, four *Leghum* species, and four *Gramineae* species were selected for host specificity testing using leaf bioassays. The procedure to determine the response of the compounds was as described above.

Root growth inhibition. A single batch of roots of the white clover (*T. repens*) from natural agricultural white clover populations from NYAES was used in this experiment. Seedlings having primary roots with three or five leaves were selected and placed in 9 cm diameter Petri dishes (3 roots/dish). Dishes contained 10 ml of the toxin preparation diluted with 2% aqueous ethanol solution at concentrations of 0, 1.0, 5.0, 10,0, 15.0, 30.0 μg/ml. After 24 h in the dew chamber (28 ± 2°C), root death was examined. Percent of root death was obtained by comparing the rate with that of the control. There were 20 measurements of seedling root death for each treatment or control.

Results

Isolation and purification of toxins. No toxin activity was detected in the water, hexane, and chloroform fraction after partitioning with three volumes of hexane, chloroform, ethyl acetate, and butanol (Table 1). The ethyl acetate, butanol extract contained two different compounds, including herbicidal and antibiotic toxin fractions, detected by bioassay. The two toxin fractions were separated by using linear gradient solvent system initiated with 15% methanol to 85% methanol for 50 min with monitoring at 254 nm under these RP-HPLC conditions (Fig. 3).

These two fractions were eluted separately and then rechromatographed. By using different solvent systems, the toxin fractions were detected with different retention time, with compounds I and II eluted at 3.49 and 4.13 min, respectively (Fig. 5). Visually, compound I appeared as a dark-brown liquid, while compound II as a yellow liquid.

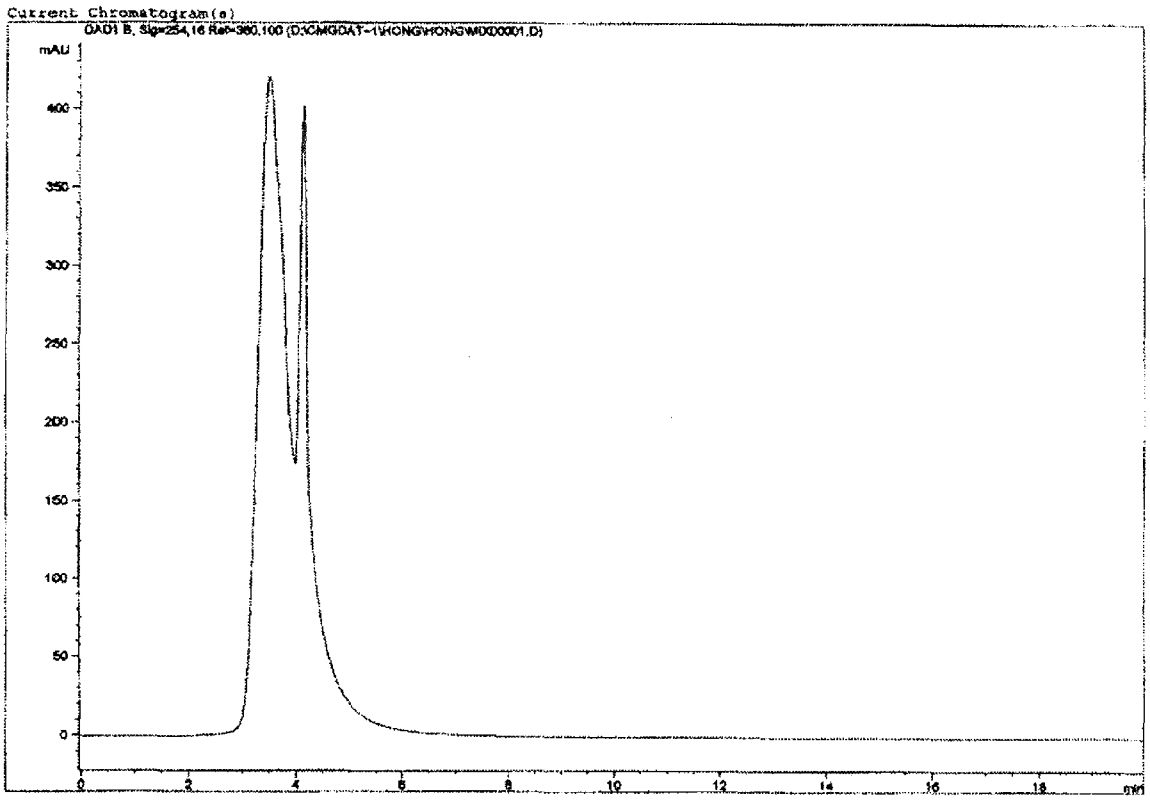

Fig. 3. HPLC chromatogram of EtOAc and BuOH fraction of BWC98-105 isolate *Sclerotinia* sp.

Table 1. Evaluation of toxicity of culture filtrates to *T. repens* by using linear separation system

Fraction	Response of Toxicity[a]
n-Butanol	+[b]
Ethyl Acetate	+
Chloroform	−
n-Hexane	−
2 % Ethanol	−
Distilled Water	−

[a]The basal portion of a leaf was covered by bending it with cotton (10 × 10 × 0.5 mm) soaked with each concentrated component containing 1.5 μg/ml of the toxin in 2% aqueous ethanol with 0.05% Tween 20, and then by placing it in a dew chamber for 24 h to keep the leaf from drying. Leaf moistened with 2% aqueous ethanol with cotton was used as control.

[b]+ = symptoms produced; − = no symptom developed.

Both toxins were highly soluble in chloroform, and were fairly stable to heat. Neither autoclaving at 121°C for 15 min, nor storing at room temperature affected both toxins.

Phytotoxicity of toxins. Compound I appeared to have antibiotic activity to phytopathogenic bacteria, but compound II showed typical herbicidal activity. In the leaf bioassay using compound II, first symptoms appeared within 4 h as water soaked rot which subsequently developed into well-defined typical toxin response surrounding black lesions.

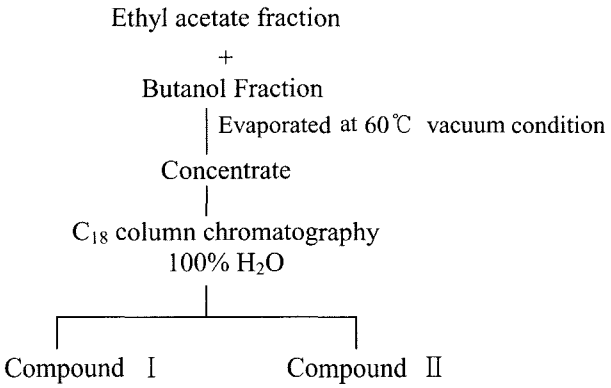

Fig. 4. Isolation procedure of compounds I and II isolated from EtOAc and BuOH fraction of BWC98-105 isolate *Sclerotinia* sp.

Symptoms on detached *T. repens* leaves produced by the compound were similar to those produced by the mycelia of the pathogen after 24 h. Compound II qualitatively induced the same symptoms as that of mycelia produced (Fig. 6).

Host specificity of toxins. Compound II showed strong phytotoxicity to *Leghum* sp. like *T. repens,* and to Indian joint-vetch and other slightly *Leghum* sp. However, it did not show phytotoxicity to other hosts tested such as soybean (Table 3). Compound II induced typical symptoms on the leaves of plants susceptible to the fungus, but did not

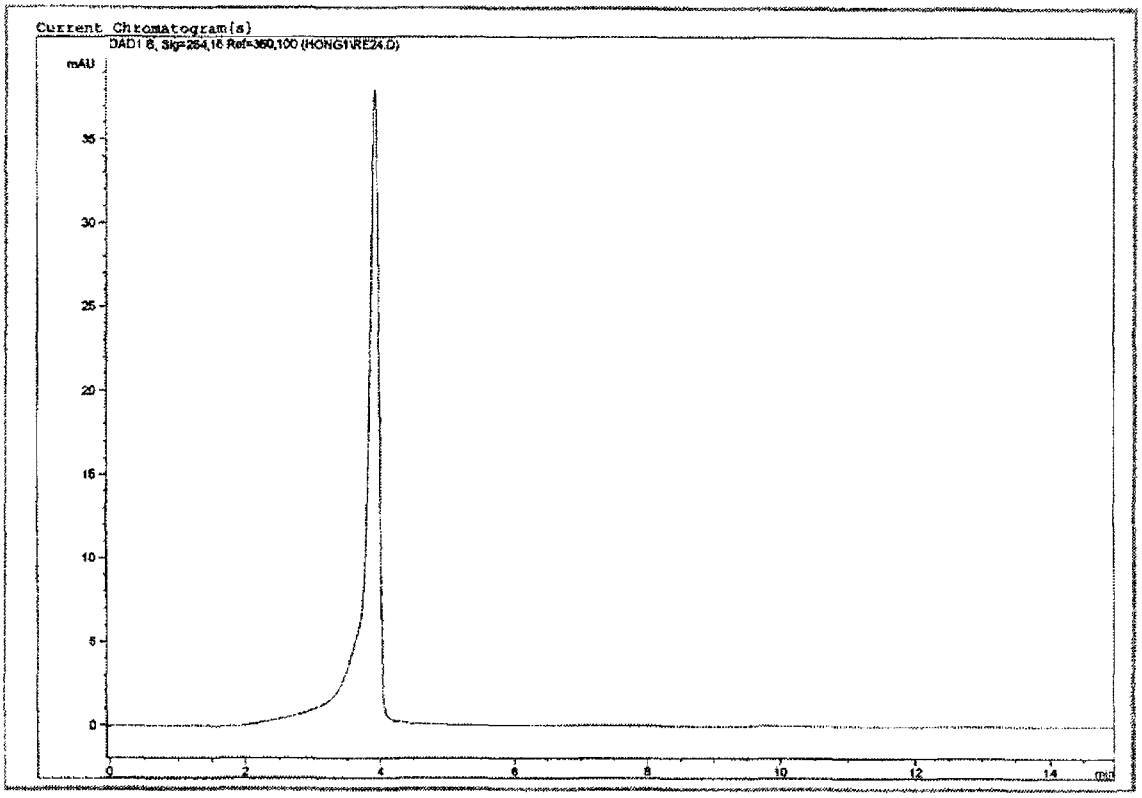

Fig. 5. HPLC chromatogram of isolated compound II of BWC98-105 isolate *Sclerotinia* sp.

Fig. 6. Effect of compound (1.5 μg/ml) on white clover (*Trifoliorum repens*). The plants on the left as control were inoculated with 2% ethanol only, while those on the right were sprayed with 20 ml of compound. The photograph was made 4 days after the plants were inoculated.

produce any effects on non-hosts. Symptom expression on *T. repens* with compound II was much stronger.

Root growth inhibition. Root growth of *T. repens* was susceptible to compound II, but not that of rice. Concentrations of 0.5 μg/ml of toxin inhibited *T. repens* root growth by 80% (Table 2). Similarly, the 30 μg/ml compound II treatment inhibited *T. repens* root growth by approximately 100%, whereas 2% ethanol root growth was inhibited by less than 0.5% by this treatment.

Table 2. Root growth inhibition rate of purified fraction compound II on *T. repens*

Concentration (μg/ml)	Percent of plant (root) death
1.0	80.3 b
5.0	85.2 b
10.0	95.6 bc
15.0	97.2 c
30.0	100.0 c
Control (2% ethanol)	0.5 a

[a]The basal portion of a leaf was covered by bending it with cotton (10 × 10 × 0.5 mm) soaked with each concentrated component containing 1.5 μg/ml of the toxin in 2% aqueous ethanol with 0.05% Tween 20, and then by placing it in a dew chamber for 24 h to keep the leaf from drying. Leaf moistened with 2% aqueous ethanol with cotton was used as control. Data were collected 10 days after inoculation.

[b]Data were processed by SAS GLM procedure (IRRI) and analyzed by Duncan's multiple range test.

Discussion

Compound I appeared to have an antibiotic activity to phytopathogenic bacteria but compound II showed a typical herbicidal activity. The toxins also had phytotoxic properties

Table 3. Response of some *Gramineae* sp., lawn grasses, and *Leguminosae* sp. to inoculation with compound II (1.5 μg/ml) of BWC98-105 from *Trifoliorum repens*

Plant[a]	Toxicity[b]
Rice (*Oryza sativa* L.)	–
Barley (*Hordeum vulgare* L.)	–
Maize (*Zea mays* L.)	–
Wheat (*Triticum aestivum* L.)	–
Soybean (*Glycine max* (L.) Merrill)	+
Penut (*Arachis hypogaea* L.)	+
Green pea (*Pisum sativum* L.)	
Cow pea (*Vigna sinensis* K.)	
White clover (*T. repens*)	++
Indian joint-vetch (*Aeshynomene indica* L.)	++
Creeping bentgrass (*Agrostis palustris* Huds.)	–
Kenterky bluegrass (*Poa pratensis* L.)	–
Perennial ryegrass (*Lolium perenne* L.) Zoysiagrass	–
(*Zoysia japonica* Steud.)	+

[a] Host plants of three or five leaf stages were covered by bending them with cotton (10 × 10 × 0.5 mm) soaked with each concentrated component containing 1.5 μg/ml of the toxin in 2% aqueous ethanol with 0.05% Tween 20, and then by placing them in a dew chamber for 24 h to keep the leaves from drying. Leaves moistened with 2% aqueous ethanol with cotton were used as control. Data were collected 10 days after inoculation.

[b] + = symptoms produced; ++ = heavily infected; = slight change in color; – = no symptom developed.

towards *Leghum* sp. besides the *Gramineae* sp. Compound I might be a novel antibiotic that is highly active on phytopathogenic bacteria. However, further research is necessary to be able to properly identify these toxins. A similar result has shown that the first chemical substance isolated from *E. monoceras* culture was monocerin. This substance was not characterized as a phytotoxic mycotoxin but was described as an antibiotic to protect wheat (*Tritichum aestivum* L.) against powdery mildew (*Erisiphe graminis* D.C.) (Zhang et al., 2000). Pringle and Scheffer (1964) reported that the host-specific 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. There has been considerable research interest in phytotoxic mycotoxins produced by plant pathogens of crop plants. Zhang et al. (2000) also extracted toxins I and II from *Exserohilum monoceras,* and evaluated them as potential bioherbicides for the control of *Echinochloa* sp. Bioassays have shown that *E. monoceras* produced phytotoxic mycotoxins biologically active against *Echinochloa* species. Two biologically active compounds, designated as toxin I and toxin II, were isolated from *E. monoceras* culture filtrate and inoculated onto plant leaves by means of extraction. In the results, toxin I was found to be the most potent and host-specific. In several instances, these phytotoxic mycotoxins have proven to be useful as tools for screening plants for toxin insensitivity (resistance) and as probes of normal physiological plant function. However, phytotoxic mycotoxins 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 *Gramineae* sp. and *T. repens* plants suggests that the toxin has the potential to be used as one of the building blocks for novel herbicides.

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