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Herbicidal Potential of Ophiobolins Produced by *Drechslera gigantea*

ANTONIO EVIDENTE,*,† ANNA ANDOLFI,† ALESSIO CIMMINO,† MAURIZIO VURRO,‡ MARIANO FRACCHIOLLA,§ AND RAGHAVAN CHARUDATTAN||

Dipartimento di Scienze del Suolo della Pianta e dell'Ambiente, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy, Istituto di Scienze delle Produzioni Alimentari, CNR, Via Amendola 122/d, 70125 Bari, Italy, Dipartimento di Scienze delle Produzioni Vegetali, Università di Bari, Via Amendola 165/a, 70125 Bari, Italy, and Plant Pathology Department, University of Florida/IFAS, Gainesville, Florida 32611-0680

Drechslera gigantea, a potential mycoherbicide of grass weeds, was isolated in Florida from naturally infected large crabgrass (*Digitaria sanguinalis*); it produces phytotoxic metabolites in liquid culture. The main metabolite was identified by spectroscopic methods and optical properties as ophiobolin A (**1**), a well-known phytotoxic sesterterpene produced by several phytopathogenic fungi of important crops and already extensively studied for its interesting biological activities. The other three minor metabolites proved to be related to ophiobolin A and were identified using the same techniques as 6-*epi*-ophiobolin A and 3-anhydro-6-*epi*-ophiobolin A (**2** and **3**) and ophiobolin I (**4**). Assayed on punctured detached leaves of several grass and dicotyledon weeds, ophiobolin A proved to be on average more phytotoxic as compared to the other related compounds. Some structural features appear to be important for the phytoxicity, such as the hydroxy group at C-3, the stereochemistry at C-6, and the aldehyde group at C-7. Furthermore, grass weeds usually proved to be more sensitive to the phytotoxins than dicotyledons, on which ophiobolin A caused the appearance of large necrosis even at the lowest concentration assayed. This is the first report about the production of ophiobolins from *D. gigantea* and of the proposed use as potential natural herbicides against grass weeds.

KEYWORDS: Large crabgrass; *Digitaria sanguinalis*; *Drechslera gigantea*; phytotoxins; ophiobolins

INTRODUCTION

Drechslera gigantea Heald & Wolf is a cosmopolitan fungal pathogen found throughout North and South America, Japan, and other regions (*1*). It causes a zonate eye spot disease of grasses, banana, and coconut (*2*, *3*). Under severe levels of disease, the leaf spots may coalesce, causing leaf lesions and leaf blight. Infected leaves may be killed. Chandramohan and Charudattan (*4*) and Chandramohan et al. (*5*) have shown that *D. gigantea* alone and in combination with two other grass pathogens, *Exserohilum longirostratum* and *Exserohilum rostratum*, could be used in a bioherbicide cocktail to control seven different weedy grasses. Typically, symptoms of *D. gigantea* leaf blight appear within about 1 week after the fungus is sprayed on the grass foliage and the disease progresses steadily over the following 2−3 weeks. The treated foliage is killed, and the control lasts for 10 weeks or more. Rhizomes are not killed, and the grasses will regrow after a period of mycoherbicide-induced suppression (*4*, *5*). Evaluations over the past 5 years have confirmed that these fungi are effective for grass management under field conditions (*4*, *5*). *Drechslera* is a fungal genus well known for the production of phytotoxic secondary metabolites, and most of the phytopathogenic species belonging to this genus, as well as their phytotoxins, have been widely studied as agents of very severe diseases of cereal crops (*6*−*8*). Some phytotoxins were also isolated from pathogens of grass weeds and proposed as potential natural herbicides (*9*−*11*). Recently, a novel phytotoxin named drazepinone was also identified as the major phytotoxic compound produced by a strain of *Drechslera siccans* (*12*). Considering the potential of the genus in producing bioactive metabolites and our interest in finding new phytotoxins produced by weed pathogens to be tested as new natural herbicides, it seemed of interest to investigate the production of novel metabolites by this proposed mycoherbicide. In this study, the production, isolation, and chemical and biological characterization of metabolites produced by *D. gigantea* were investigated.

MATERIALS AND METHODS

Fungus. *D. gigantea* was isolated during extensive field surveys in Florida from naturally infected large crabgrass (*Digitaria sanguinalis*) (*4*). It was stored on a potato dextrose agar (PDA) slant in the Biological

* To whom correspondence should be addressed. Tel: +39-081-2539178. Fax: +39-081-2539186. E-mail: evidente@unina.it.
† Università di Napoli Federico II.
‡ CNR.
§ Università di Bari.
|| University of Florida.

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Control of Weeds Collection at the Plant Pathology Department, University of Florida/IFAS (Gainesville, FL) (N. LCLF-1).

General Experimental Procedures. Optical rotations were measured in $CHCl_3$ solution on a Jasco (Tokyo, Japan) P-1010 digital polarimeter; infrared (IR) spectra were recorded as neat on a Perkin-Elmer (Norwalk, CT) Spectrum One Fourier transform-IR spectrometer, and UV spectra were taken in CH_3CN solution on a Perkin-Elmer Lambda 25 UV/vis spectrophotometer. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 500 or 400 MHz and at 125, 100, or 75 MHz, respectively, in $CDCl_3$, on Bruker (Karlsruhe, Germany) spectrometers. The same solvent was used as the internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra (*13*). DEPT and correlated spectroscopy experiments (*13*) were performed using Bruker microprograms. Electron ionization (EI) spectra were taken at 70 eV on a Fisons (Berkely, MA) Trio-2000 spectrometer; electrospray ionization (ESI) MS were recorded on a Perkin-Elmer API 100 liquid chromatography-MS with a probe voltage of 5300 V and a declustering potential of 50 V. Analytical and preparative thin-layer chromatography (TLC) were performed on Kieselgel 60 F_{254}, 0.25 and 0.50 mm of thickness, silica gel plates (Merck, Darmstadt, Germany), respectively; the spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min. For column chromatography (CC), Kieselgel 60, 0.063−0.20 mm silica gel (Merck), was used. The X-ray analysis of ophiobolin A was carried out on a colorless crystal obtained from benzene solution (slow evaporation). Data were acquired on a Nonius Mch3 (Delft, Holland) single-crystal diffratometer (graphite-monochromated Mo Kα radiation). The collection of the data and the determination of the structure were performed by Pr. A. Tuzi of Dipartimento di Chimica, Università di Napoli Federico II, and will be reported elsewhere.

Production, Extraction, and Purification of *D. gigantea* Ophiobolins. The fungus was grown and maintained on Petri dishes containing PDA (Oxoid, England). For the production of phytotoxic metabolites, flasks (1 L) containing a mineral-defined medium (350 mL) (*14*) were seeded with mycelium fragments obtained from colonies actively growing on PDA. The cultures were incubated under shaken conditions (100 rpm) at 25 °C in the dark for 8 days, then filtered, assayed for phytotoxic activity, and lyophilized for the successive purification steps. The lyophilized material obtained from the culture filtrates (2.7 L) was dissolved in distilled water (300 mL, final pH 4.2) and extracted with ethyl acetate (3 × 300 mL). The organic extracts were combined, dehydrated with Na_2SO_4, filtered, and evaporated under reduced pressure. The brown oil residue (393.5 mg) proved to be highly phytotoxic when assayed as described before on detached leaves of *Phalaris canariensis*. It was fractionated by CC eluted with $CHCl_3$−*iso*-PrOH (96:4, v/v) yielding 10 groups of homogeneous fractions (1.3, 3.4, 149.0, 24.4, 2.6, 5.7, 26.0, 5.5, 9.3, and 69.2 mg). The last fraction was eluted with methanol. The residue of the third fraction (149.0 mg) was crystallized three times with ethyl acetate−*n*-hexane (1:5) and gave the main metabolite (43 mg) as a white crystal. The pure metabolite was identified as ophiobolin A (**1**; **Figure 1**). The residues obtained from the mother liquors of ophiobolin A crystallization (55 mg) were purified by preparative TLC [eluent EtOAC−*n*-hexane (5.5:4.5, v/v)] producing three bands. The first of them (R_f 0.55, 45 mg) was further purified by preparative TLC [$CHCl_3$−*iso*-PrOH (96:4, v/v)] yielding a further amount of ophiobolin A (24.5 mg) as a white crystalline solid, for a total of 67.5 mg (25.0 mg/L), and 6-*epi*-ophiobolin A (**2**; **Figure 1**) (R_f 0.32, 4.1 mg, 1.5 mg/L). The second band of the first TLC (R_f 0.66) appeared to be a homogeneous amorphous solid [R_f 0.62, eluent $CHCl_3$−*iso*-PrOH (96:4, v/v), 3.0 mg, 1.1 mg/L] and was identified as 3-anhydro-6-*epi*-ophiobolin A (**3**; **Figure 1**). The residue of the seventh fraction (26.0 mg) of the first column, containing ophiobolin A and another metabolite, was further purified by two successive preparative TLC steps [EtOAC−*n*-hexane (5.5:4.5, v/v) and $CHCl_3$−*iso*-PrOH (94:6, v/v)], yielding a further amount of the main metabolite (**1**) (2.43 mg, for a total of 69.9 mg, 25.9 mg/L) and another amorphous solid identified as ophiobolin I (**4**; **Figure 1**) (R_f 0.20, eluent 0.8 mg, 0.3 mg/L).

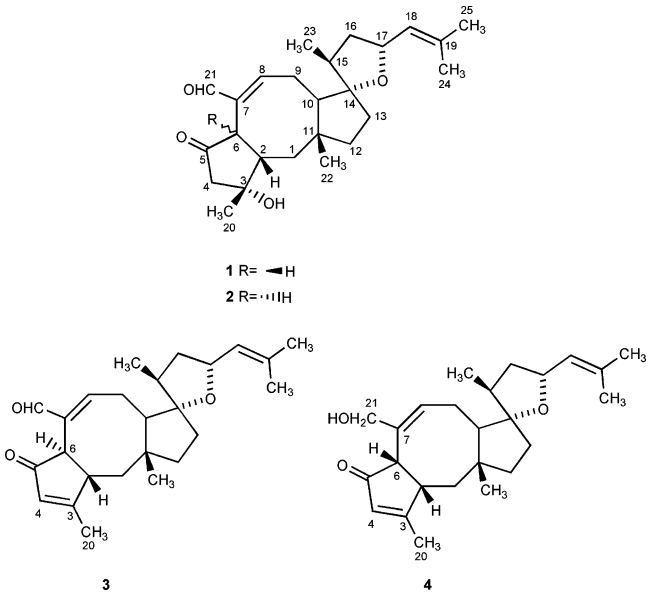

Figure 1. Structures of ophiobolin A, 6-*epi*-ohpiobolin A, 3-anhydro-6-*epi*-ophiobolin A, and ophiobolin I (**1**–**4**, respectively).

Biological Assay. For the preliminary assessment of phytotoxicity, culture filtrates, organic extract, chromatographic fractions, and pure ophiobolins were assayed by using a leaf assay on *P. canariensis* plants. Pure metabolites, as well as crude extract and fractions, were first dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted to the desired final concentrations with distilled water (final concentration of DMSO, 2%). Droplets (8 μL) of the assay solutions were applied to cut segments (length around 5 cm) of leaves detached from young plants grown in greenhouse conditions, on which circular superficial lesions (0.5 mm) have previously been produced by using a glass capillary pipet. After droplet application, leaf segments were kept on moistened paper filters in Petri dishes, in a growth cabinet at 25 °C under continuous fluorescent lights (10000 lux). Droplets of DMSO solution (up to 4%) were applied to leaves as controls. Symptom appearance was observed 2 days after droplet application. Using the procedure described, all of the pure ophiobolins were tested at 50, 100, and 250 μg/mL on 13 weed species (eight mono- and five dicotyledons) listed in **Table 1**. Symptoms were evaluated using a visual empirical scale from 0 (no symptoms) to 3 (diameter of the necrotic area, 3 mm or wider).

Ophiobolin A (1). White crystals; mp 182−185 °C; $[\alpha]_D^{25}$ +270° (*c* 0.4). IR ν_{max}: 3468, 1740, 1664 cm^{-1}. UV λ_{max} nm (log ϵ): 238 (4.1). Literature values (*15*): mp 182 °C; $[\alpha]_D$ +270°. IR ν_{max} ($CHCl_3$): 3500, 1743, 1633 cm^{-1}. UV λ_{max} (EtOH) nm (ϵ): 238 (13800). Literature values (*16*): mp 170−172 °C; $[\alpha]_D$ +265.5° (*c* 1.0, $CHCl_3$). IR ν_{max}: 3500, 1730, 1690, 1660, 1625 cm^{-1}. ^1H NMR spectrum was integrated in respect that it was already reported (*16*, *17*) for the following signals, δ: 2.04 and 1.37 (1H each, m, H_2C-13), 1.41 (1H, dd, J = 12.0 and 3.8 Hz, H-12A). The ^{13}C NMR spectrum was very similar in that it was already reported (*16*). EI-MS *m*/*z* (rel int.): 401 $[M + H]^+$ (11), 383 $[M + H - H_2O]^+$ (15), 319 $[M + H - C_6H_{10}]^+$ (28), 300 $[M - C_6H_{10} - H_2O]^+$ (33), 273 $[M - C_8H_{13} - H_2O]^+$ (32), 164 (100). ESI-MS (+) *m*/*z*: 401 $[M + H]^+$, 423 $[M + Na]^+$, 439 $[M + K]^+$.

6-*epi*-Ophiobolin A (2). Amorphous solid; $[\alpha]_D^{25}$ +44° (*c* 0.1). Literature values (*18*): $[\alpha]_D$ +46° (*c* 5.3, $CHCl_3$). IR ν_{max}: 3445, 1742, 1683 cm^{-1}. Literature values (*19*): IR ν_{max} film: 3450, 1740, 1684, 1640 cm^{-1}. UV λ_{max} nm (log ϵ): 235 (4.0). The ^1H NMR spectrum was integrated with respect to those already reported (*17*, *18*) for the following signals, δ: 2.18 (1H, br q, H-15), 1.80 and 1.40 (1H, each, m, H_2C-13). The ^{13}C NMR spectrum was very similar to that already reported (*20*). EI-MS *m*/*z*: 401 $[M + H]^+$ (1), 383 $[M + H - H_2O]^+$ (2), 319 $[M + H - C_6H_{10}]^+$ (2), 300 $[M - C_6H_{10} - H_2O]^+$ (3), 273 $[M - C_8H_{13} - H_2O]^+$ (3), 164 (40), 107 (100). Literature values (*18*): EI *m*/*z*: 400 $(M)^+$, 382, 273, 176, 165. ESI-MS (+) *m*/*z*: 401 $[M + H]^+$, 423 $[M + Na]^+$, 439 $[M + K]^+$.

Table 1. Effect of **1**–**3** on Various Weed Species Tested by Leaf Puncture Assay[a]

	compound								
	1			**2**			**3**		
	concentration (M)								
species	6.3×10^{-4}	2.5×10^{-4}	1.3×10^{-4}	6.3×10^{-4}	2.5×10^{-4}	1.3×10^{-4}	6.6×10^{-4}	2.6×10^{-4}	1.3×10^{-4}
monocotyledons									
Avena ludoviciana Dur.	3	3	3	2	2	2	0	0	0
Bromus sterilis L.	3	2	1	2	1	1	1	1	1
C. dactylon (L.) Pers.	1	0	0	2	1	0	0	0	0
D. sanguinalis (L.) Scop.	3	3	3	3	2	2	1	1	0
Echinochloa crus-galli (L.) Beauv.	3	3	3	2	2	1	1	1	1
Oryzopsis miliacea (L.) Aschers	3	2	1	2	1	1	1	1	1
P. canariensis L.	3	3	3	3	2	1	1	1	1
S. viridis (L.) Beauv.	3	3	3	3	3	3	2	2	2
dicotyledons									
Amaranthus retroflexus L.	3	2	2	1	1	1	0	0	0
Chenopodium album L.	3	2	2	3	2	2	1	1	0
Convolvulus arvensis L.	2	2	2	2	2	2	0	0	0
D. erucoides (L.) DC	2	2	2	2	2	2	3	3	2
S. oleraceus L.	3	3	3	3	3	3	1	1	0

[a] Observations were made 2 days after treatment. Diameter of necrosis on leaves: 3, necrosis > 3 mm; 2, necrosis between 2 and 3 mm; 1, necrosis between 1 and 2 mm; and 0, no necrosis.

3-Anhydro-6-*epi*-ophiobolin A (3). Amorphous solid; $[\alpha]_D^{25}$ +7 (*c* 0.1). IR ν_{max}: 1685, 1645 cm[1]. UV λ_{max} nm (log ϵ): 225 (3.6), 235 (4.0). The ^{1}H NMR spectrum was integrated with respect to those already reported (*17*, *18*) for the following signals, δ: 2.24 (1H, dd, J = 6.7 and 3.8 Hz, H-15), 1.99 (1H, dd, J = 13.2, 2.5 Hz, H-13A), 1.80 (H, m, H-12A), 1.42 and 1.77 (1H each, m H_2C-1). The ^{13}C NMR spectrum was very similar to that already reported (*19*). EI-MS *m*/*z*: 383 $[M + H]^+$ (27), 301 $[M + H - C_6H_{10}]^+$ (45), 273 $[M - C_8H_{13}]^+$ (3), 175 (100). ESI-MS (+) *m*/*z*: 383 $[M + H]^+$, 405 $[M + Na]^+$, 421 $[M + K]^+$.

Ophiobolin I (4). $[\alpha]_D^{25}$ +46.7 (*c* 0.2). IR ν_{max}: 3409, 1682, 1619 cm^{-1}. Literature values (*16*): $[\alpha]_D^{25}$ +48.6° (*c* 1.0, $CHCl_3$). IR ν_{max}: 3450, 1680, 1657, 1613 cm^{-1}. UV λ_{max} nm (log ϵ): 225 (4.3). The ^{1}H NMR spectrum was very similar to those previously reported (*16*, *18*, *20*). ESI-MS *m*/*z*: 385 $[M + H]^+$, 407 $[M + Na]^+$, 423 $[M + K]^+$. Literature values (*20*): EIHRMS $C_{25}H_{36}O_3$ $[M^+$; obsd *m*/*z*: 384.2665 $(M)^+$, 366.2559].

RESULTS AND DISCUSSION

Extraction and Purification of Ophiobolins. The organic extract showing a strong phytotoxic activity was purified by column chromatography and TLC. The main metabolite was isolated as a crystalline white solid (25.0 mg/L) and identified by spectroscopic methods (essentially ^{1}H and ^{13}C NMR and MS techniques) as ophiobolin A (**1**; **Figure 1**). The physical and the spectroscopic data were similar to those previously reported in the literature (*15*−*17*). This result was also confirmed by a direct X-ray analysis carried out on the natural metabolite. The crystallographic data will be reported elsewhere, as only X-ray analysis of methoxybromide (*21*) or the tetrahydro (*22*) derivatives of ophiobolin A has been described previously. The other three compounds were isolated as amorphous solids but in lower amounts (1.5, 1.1, and 0.3 mg/L for **2**−**4**, respectively) as compared to **1** and appeared to be closely related to ophiobolin A by preliminary spectroscopic investigation. They were identified by comparison of their spectroscopic data, essentially ^{1}H and ^{13}C NMR and MS data, as 6-*epi*-ophiobolin A, 3-anhydro-6-*epi*-ophiobolin A, and ophiobolin I (**2**, **3**, and **4**; **Figure 1**). Their physical and the spectroscopic data were similar to those reported in the literature (*16*−*20*).

Biological Activity. Ophiobolin A proved to be highly phytotoxic to almost all of the plant species tested (**Table 1**), even at the lowest concentration used (1.3×10^{-4} M; 50 μg/mL). Among dicotyledons, *Sonchus oleraceus* L. appeared to be particularly sensitive, whereas almost all of the monocotyledons were very sensitive. In contrast, even at the highest concentration used, the phytotoxin was almost inactive to *Cynodon dactylon* (L.) Pers. As compared to ophiobolin A, 6-*epi*-ophiobolin A proved to have almost the same spectrum of plant sensitivity but at a lower intensity. With regard to 3-anhydro-6-*epi*-ophiobolin A, it was almost inactive on most of the plants tested, with the exception of *Setaria viridis* (L.) Beauv. and *Diplotaxis erucoides* (L.) DC. Ophiobolin I proved to be inactive, even at the highest concentration, to all of the plants tested.

It is interesting to note a certain plant level of selectivity of the phytotoxins. In fact, on average, the tested ophiobolins proved to be more active on grass weeds relative to dicotyledons species. Studies are in progress in order to evaluate the ability of the compounds to penetrate in the tissue without injuries or using surfactants and adhesivants that could facilitate absorption.

The ophiobolins are a group of polycyclic sesterterpenoids with a common moiety. They are secondary phytotoxic metabolites produced by pathogenic fungi attacking several crops, such as rice, maize, and sorghum. Ophiobolin A was the first member of the group to be isolated and characterized independently by Canonica et al. (*23*) and Nozoe et al. (*24*). In addition to ophiobolin A, several analogues were isolated in the late 1960s and their structures were determined. These include ophiobolin B from *Bipolaris oryzae* (*25*), ophiobolin C from *Bipolaris zizanie* (*24*), ophiobolin D from *Cephalosporium caerulens* (*26*, *27*), and ophiobolin F from *Bipolaris maydis* (*28*). A wealth of information has been accumulated regarding the biological activities of ophiobolins as well as their biosynthesis, although neither the enzymes nor the genes responsible have been identified (*29*). They share the same carbotrycyclic diterpenoid ring with fucicoccins and cotylenins, two other groups of microbial metabolites produced by *Fusicoccum amygdali*, the causal agent of almond and peach diseases (*30*), and by *Cladosporium* sp. 501-7W (*31*, *32*). Although ophiobolins were quite heavily studied for their interesting effects on plant physiology and for their biological activities, only limited information has been reported on their potential herbicidal

activity. Furthermore, there are only a few studies on structure−activity relationships. One of them was carried out on four ophiobolins, namely, **1**−**3** and 3-anhydroophiobolin A, isolated from the culture filtrates of the *Bipolaris sorghicola*, a fungal pathogen of Johnsongrass (*Sorghum halepense* L.), using a leaf spot assay on several plants. The results showed that ophiobolin A and its 6-epimer were more phytotoxic than their anhydro derivatives against sorghum, sicklepod, and maize. 6-*epi*-Ophiobolin A at a high concentration produced the largest necrosis on leaves of all plants tested except morning glory. The anhydrous derivatives were generally less and not phytotoxic at all to morning glory leaves, even at concentrations of 2 mg/mL (*33*). In our assays, ophiobolin A proved to be more active on almost all of the plant species tested in comparison with 6-*epi*-ophiobolin A, whereas the 3-anhydro compound, in agreement to the previuosly reported data (*33*), was much less phytotoxic, being almost inactive on many of the plants tested, even at the highest concentration used. Furthermore, ophiobolin I proved to be inactive to all of the species tested. On the basis of these results, structural features important for the phytotoxicity appear to be the hydroxy group at C-3, the stereochemistry at C-6, and the aldehyde group at C-7.

Conclusions. The isolation of ophiobolins from this strain of *D. gigantea* isolated from *D. sanguinalis* is surprising considering that Kenfield et al. (*10*) had previously studied the metabolites produced by another strain of *D. gigantea* and reported only the isolation of gigantenone as the main toxin. Although both are terpenoids, gigantenone belongs to the chemical subgroup of sesquiterpenes, whereas ophiobolins belong to that of sesterterpenoids. Several biological investigations have also described gigantenone as a promising compound in different areas of research such as pathological physiology, photosynthetic efficiency, senescence, vegetation propagation, and development of selective herbicides (*11*). Many biological properties were also reported for ophiobolins. For example, they can reduce root and coleoptile growth of wheat seedlings, inhibit seed germination, change cell membrane permeability, stimulate leakage of electrolytes and glucose, or cause respiratory changes (*29*). In our assays, the necrotic spot lesions on leaves induced by the application of drops of phytotoxins resemble those caused by the pathogen, even if these symptoms are not as specific as the pathogen. For this reason, further studies are in progress to evaluate the possibility of enhancing the efficacy of the promising mycoherbicide *D. gigantea* with the joint application of sublethal doses of the pytotoxins.

Changes in culturing conditions can strongly influence the biosynthetic production of ophiobolins. For example, *B. maydis* was able to produce ophiobolin A, 3-anhydroophiobolin A, ophiobolin B, and ophiobolin L when grown in liquid conditions (*16*), whereas it produced ophiobolin M, 6-*epi*-ophiobolin M, ophiobolin C, 6-*epi*-ophiobolin C, ophiobolin K, and 6-*epi*-ophiobolin K when grown on solid media (*34*). For this reason, further studies are in progress to obtain novel ophiobolins by adjustments of the culturing condition of our strain of *D. gigantea*.

Ophiobolins are also toxic to animals and nematodes (*34*). For example, the LD_{50} values of ophiobolin A for mice are 238 and 73 mg/kg when administered subcutaneously or orally, respectively (*35*). Even if they are much less acutely toxic as compared to other powerful mycotoxins [e.g., oral LD_{50} for T-2 toxin and aflatoxin B_1 ranges between 0.6 and 6.1 mg/kg and between 0.4 and 18 mg/kg, respectively, depending on the animal species (*36*)], their real impact in the environment should be evaluated, as well as their effect on nontarget organisms, and their fate after the introduction in the environment, if considered as possible natural herbicides. Furthermore, new ophiobolins could be isolated from other fungal strains, or new derivatives could be obtained starting from the natural compounds, having a lower toxicity or stronger phytotoxic properties.

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