Antifungal Activity against Plant Pathogens of Metabolites from the Endophytic Fungus Cladosporium cladosporioides

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Antifungal Activity against Plant Pathogens of Metabolites from the Endophytic Fungus *Cladosporium cladosporioides*

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Supporting Information

ABSTRACT: Bioassay-guided fractionation of *Cladosporium cladosporioides* (Fresen.) de Vries extracts led to the isolation of four compounds, including cladosporin, **1**; isocladosporin, **2**; 5′-hydroxyasperentin, **3**; and cladosporin-8-methyl ether, **4**. An additional compound, 5′,6-diacetylcladosporin, **5**, was synthesized by acetylation of compound **3**. Compounds **1**−**5** were evaluated for antifungal activity against plant pathogens. *Phomopsis viticola* was the most sensitive fungus to the tested compounds. At 30 μM, compound **1** exhibited 92.7, 90.1, 95.4, and 79.9% growth inhibition against *Colletotrichum acutatum*, *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, and *P. viticola*, respectively. Compound **2** showed 50.4, 60.2, and 83.0% growth inhibition at 30 μM against *Co. fragariae*, *Co. gloeosporioides*, and *P. viticola*, respectively. Compounds **3** and **4** were isolated for the first time from *Cl. cladosporioides*. Moreover, the identification of essential structural features of the cladosporin nuclei has also been evaluated. These structures provide new templates for the potential treatment and management of plant diseases.

KEYWORDS: *Cladosporium cladosporioides, antifungal, Colletotrichum species, Phomopsis species, cladosporin, anthracnose*

INTRODUCTION

Strawberry anthracnose, a serious disease in many parts of the world, is caused by filamentous fungi of the genus *Colletotrichum*. *Colletotrichum* species are defined as destructive pathogens that cause significant economic damage to crops worldwide. The three most common *Colletotrichum* species on strawberry (*Fragaria* × *ananassa* Duchesne) are *Colletotrichum fragariae* A. N. Brooks, *Colletotrichum acutatum* J. H. Simmonds, and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., which may cause anthracnose disease either singularly or in combination.[1,2] Strawberry anthracnose can be devastating because other plant parts may be infected in addition to the fruit. This infection results in millions of dollars in crop loss each year.[3] *Phomopsis viticola* Sacc. is another common plant pathogen that causes severe diseases in grapes such as phomopsis cane and leaf spot disease. *Phomopsis obscurans* causes leaf blight of the cultivated strawberry and can also infect foliage, runners, petioles, and fruit with a dark-brown center surrounded by light-brown rings with purplish halos.[4,5]

Increasing incidence of chemical resistance in fungal pathogens and potential environmental and mammalian toxicities, often caused by the application of conventional fungicides, are factors that drive a need to search for new safe plant protectants.[6] It is particularly desirable to evaluate biologically active natural products possessing new chemical classes that might function by modes of action different from those of existing fungicides, thus avoiding problems of cross-resistance to current chemical classes. In terms of the availability of the starting materials, endophytic fungi provide a rich source of numerous agrochemical agents.[7] In the course of discovery of new pest-control agents from fungi as alternatives to synthetic molecules, 40 fungal crude extracts were screened using a direct bioautography coupled with *Colletotrichum* as the detection method. Acetone extracts of *Cladosporium cladosporioides* (Fresen.) de Vries showed the most promising activity against the three *Colletotrichum* species and was selected for further in-depth studies. *Cl. cladosporioides* is a very common saprophytic fungus, but it is also a pathogen of many different host plants. It can be isolated from many sources including air, soil, textiles, and several other substrates.[8] *Cl. cladosporioides* has been reported to be very effective both in the prevention and as a biocontrol agent of the apple scab fungus (*Venturia inaequalis*).[9]

The aim of this study was to isolate and evaluate the antifungal activity of *Cladosporium* metabolites against *Colletotrichum* species and *Phomopsis* species to develop potential agrochemical leads for disease control. This is the first report

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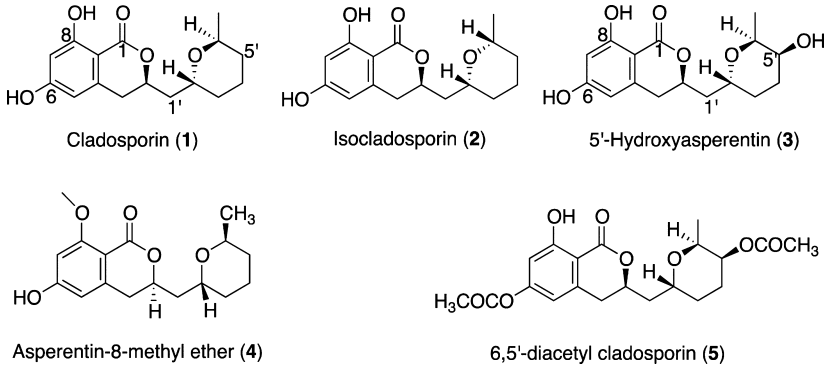

Figure 1. Compounds isolated from *Cladosporium cladosporioides*.

on the antifungal activity of *Cladosporium* metabolites against these fungus species. Moreover, the identification of essential structural features of the metabolites was also studied.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra (see the Supporting Information) were obtained on a Bruker model AMX 500 NMR spectrometer with standard pulse sequences, operating at 500 MHz in ^{1}H and 125 MHz in ^{13}C. The chemical shift values were reported in parts per million units (ppm) from trimethylsilane (TMS) using known solvent chemical shifts. Coupling constants were recorded in hertz (Hz). Standard pulse sequences were used for COSY, HMQC, HMBC, TOCSY, NOESY, and DEPT. High-resolution mass spectra (HRMS) were measured on a Micromass Q-Tof Micro mass spectrometer with a lock spray source. Column chromatography was carried out on a 25 cm × 10 cm i.d., silica gel 60 column silica gel (70−230 mesh, Merck) and Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan). TLC (silica gel 60 F254) was used to monitor fractions from column chromatography. Preparative TLC (Analtech, Newark, DE, USA) was carried out on 20 × 20 cm, 1 mm thick, silica gel 60 PF 254 + 366 plates. Visualization of the TLC plates was achieved with a UV lamp (λ_{max} 254 and 365 nm) and modified anisaldehyde spray reagent (EtOH/acetic acid/anisaldehyde/sulfuric acid, 85:9:1:5). HPLC analyses were performed on a Waters LC Module I equipped with a UV detector 486 utilizing Millennium 32 Chromatography Manager software (Waters, Milford, MA, USA). The ODS column used was a 250 × 10 mm i.d., 5 μm, Phenomenex Luna C18 column. All HPLC solvents were of HPLC grade (Fisher), filtered through appropriate membranes (water through 0.45 μm and organic solvents through 0.22 μm filters) and sparged prior to and during analysis with nitrogen at a flow rate of 50 mL/min. Chemicals for pharmacological studies were obtained from Sigma-Aldrich.

Fungal Material. *Cl. cladosporioides* fungus was collected in Tifton, GA, in 1978, lyophilized, and stored at −20 °C. The fungus was plated out on potato dextrose agar, which was maintained at 24 °C until discrete fungal colonies appeared. Then 50 mL of potato dextrose broth was inoculated with the fungus spores and incubated for 2 weeks in stationary phase at 24 °C. The fungus was subsequently seeded onto a shredded wheat medium consisting of 100 g of shredded wheat, 200 mL of low-pH mycological broth, 40 g of yeast extract, and 400 g of sucrose in a 2.0 L Fernbach flask (15 flasks were used) followed by incubation for 22 days at 24 °C.[10]

Extraction and Isolation. Following incubation, 300 mL of acetone was added to each flask (15 flasks were used), and the fungus and the substrate were homogenized. The suspension was filtered and the filtrate was concentrated under vacuum at 40 °C to yield a water fraction. The water fraction was then extracted with EtOAc (500 mL × 3). The combined EtOAc extracts were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The EtOAc extract (21 g) was chromatographed on a 25 cm × 10 cm i.d., 70−230 mesh, silica gel 60 column, with stepwise elution with hexanes, ethyl acetate, and methanol, to yield fractions A−G. Bioautography-guided bioassay showed that fractions C, D, and E exhibited antifungal activity and were selected for further bioassay-guided isolation. Fraction C was purified by crystallization from hexanes/EtOAc (1:1) to give **1** (5.04 g) (see Figure 1 for compound structures). Compound **2** (8.3 mg) was isolated by a 250 × 10 mm i.d., 5 μm, Phenomenex Luna C18 HPLC column using a MeOH/H_2O gradient elution. Fraction D was subjected to fractionation over a 40 cm × 2 cm i.d., Sephadex LH-20 CC eluted with CH_2Cl_2/MeOH (1:1) to afford 128 subfractions. Subfractions 72−128 were combined to afford compound **3** (117 mg). Subfractions 31−71 were combined and chromatographed on a 55 mm × 21 mm i.d., silica Biotage SNAP cartridge (Biotage, Charlotte, NC, USA) using a $CHCl_3$/MeOH gradient to afford **4** (3 mg). Compound **3** (50 mg) was reacted with 2 mL of acetic anhydride and 2 mL of pyridine for 24 h at room temperature, purified by preparative TLC (petroleum ether/EtOAc 1:1), and dried under nitrogen to give compound **5** (4.5 mg).

Biological Assay (See the Supporting Information). *Direct Bioautography Assay.* Bioautography procedures were described in our previous studies.[11,12] The acetone extract of *Cl. cladosporioides* was applied at 80 and 160 μg/spot in chloroform onto a silica plate. Technical fungicide grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service Inc., West Chester, PA, USA) were used as positive controls at 2 mM in 2 μL of 95% ethanol.

Microdilution Broth Assay. A standardized 96-well microdilution broth assay developed by Wedge and Kuhajek[13] was used to evaluate the antifungal activity of pure compounds from *Cl. cladosporioides* that were identified as active by bioautography.

Strains of *Co. acutatum*, *Co. fragariae*, *Co. gloeosporioides*, *Botrytis cinerea* Pers.:Fr, *Fusarium oxysporum* Schlechtend:Fr, *P. obscurans* (Ellis and Everh.) B. Sutton, and *P. viticola* Sacc. were used to evaluate the antifungal activity of the tested compounds using in vitro microdilution broth assay. Each fungus was challenged in a dose−response format using tested compounds where the final treatment concentrations were 0.3, 3.0, and 30.0 μM. Technical grade commercial fungicides captan and azoxystrobin, which represent two different modes of action, were used as positive fungicide standards. Each compound was evaluated in duplicate, and the experiment was performed three times. Mean absorbance and standard errors were used to evaluate fungal growth after 48 and 72 h except for *P. obscurans* and *P. viticola* (120 and 144 h).

RESULTS AND DISCUSSION

Bioassay-guided fractionation of *Cl. cladosporioides* crude extracts (20 g) led to the isolation of four compounds, including cladosporin, **1**; isocladosporin, **2**; 5′-hydroxyasperentin, **3**; and cladosporin-8-methyl ether, **4**. A synthesized compound, 5′,6-diacetylcladosporin, **5**, was also prepared. The structures of these compounds were established by 1D and 2D NMR spectroscopic analysis, mass spectrometric (ESI-MS) data, and X-ray crystallography, as well as comparison with the previous literatures values.[14−16] All compounds were further evaluated for their antifungal activity against seven plant

pathogens using an in vitro microdilution broth assay. In the microdilution broth assay, cladosporin, **1**, also named asperentin, caused 92.7% growth inhibition of *Co. acutatum*, 90.1% inhibition of *Co. fragariae*, and 95.4% inhibition of *Co. gloeosporioides* at 30 μM. Compound **1** is a promising compound compared to the standard fungicide azoxystrobin, which caused only 40.5% growth inhibition of *Co. acutatum* and 58.9% inhibition of *Co. fragariae* (Figure 2). *Co. acutatum* is

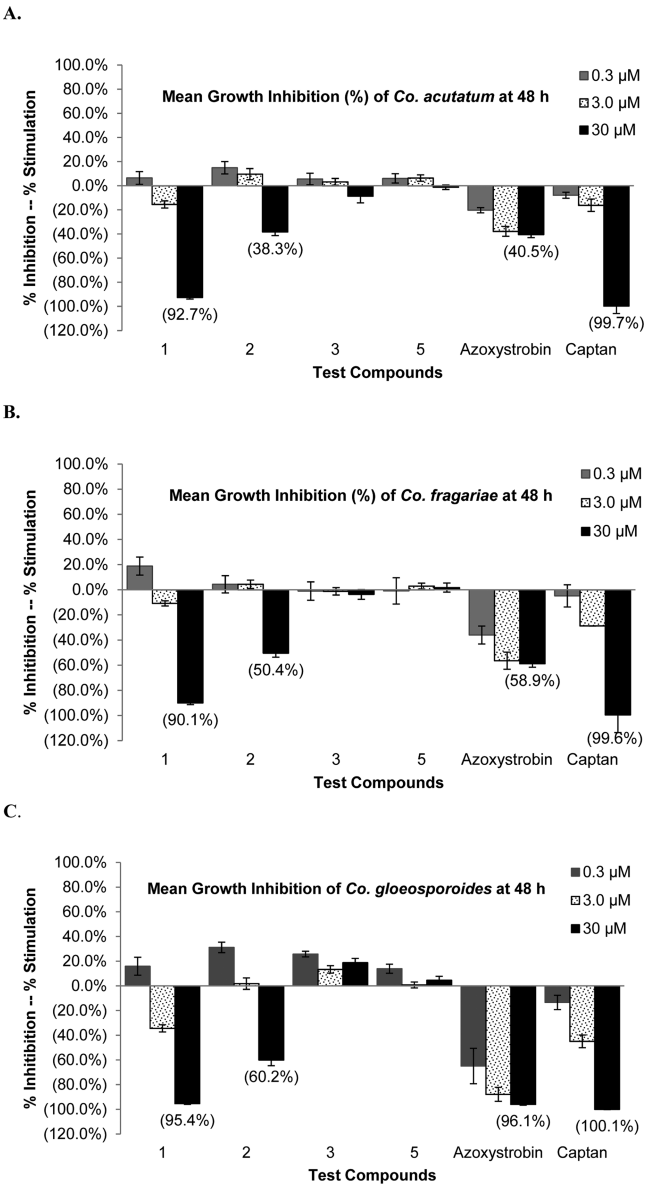

Figure 2. Mean fungal growth inhibition (%) of *Colletotrichum acutatum* (A), *Colletotrichum fragariae* (B), and *Colletotrichum gloeosporioides* (C) after exposure to compounds **1**, **2**, **3**, and **5** using a dose–response format at 48 h. Fungicide standards: captan and azoxystrobin.

genetically insensitive to the benzimidazole class of fungicides, and the activity of cladosporin in this species indicates that its mode of action is different from that of the benzimidazoles.[17,18] As shown in Figure 3, cladosporin shows significant antifungal selectivity against *P. viticola* and *P. obscurans* at 30 μM. Although this is the first report of compounds **3** and **4** from *Cl. cladosporioides*, they were previously isolated from *Aspergillus*

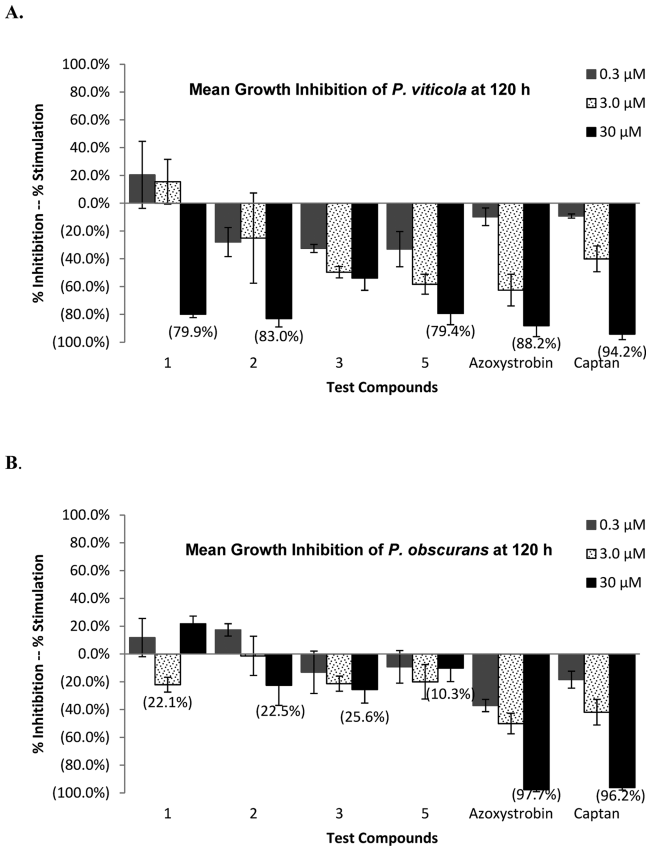

Figure 3. Mean fungal growth inhibition (%) of *Phomopsis viticola* (A) and *Phomopsis obscurans* (B) after exposure to compounds **1**, **2**, **3**, and **5** using a dose–response format at 120 h. Fungicide standards: captan and azoxystrobin.

flavus,[14] *Chaetomium globosum*,[19] and *Eurotium repens*.[20] The microdilution broth assay result demonstrated that **2** exhibited moderate antifungal activity with 50.4% growth inhibition of *Co. fragariae* and 60.2% inhibition of *Co. gloeosporioides* at 48 h (Figure 2B,C) and good antifungal selectivity against *Phomopsis* species with 80.3% growth inhibition of *P. viticola* and 22.5% inhibition of *P. obscurans* at 120 h (Figure 3A,B). As shown in Figure 3A,B, compounds **3** and **5** show no antifungal activity against the three *Colletotrichum* species, but showed good selectivity over *P. viticola* (53.9 and 79.4%, respectively) and *P. obscurans* (25.6 and 10.3%, respectively). Compound **4** had no antifungal activity against the tested fungi.

An overall evaluation of the relationship between the structures and antifungal activity of the compounds at 30 μM suggested several essential positions that might be responsible for their antifungal activity (Table 1). The absolute

Table 1. Overall Fungal Growth Inhibition (Percent) of Compounds 1, 2, 3, 4, and 5 against Plant Pathogens at 30 μM

compd	*Co. acutatum*	*Co. fragariae*	*Co. gloeosporioides*	*P. viticola*	*P. obscurans*
1	**92.7**	**90.1**	**95.4**	**79.9**	22.1
2	38.3	**50.4**	**60.2**	**83.0**	22.5
3	NA	NA	NA	**53.9**	25.6
4	NA	NA	NA	35.1	NA
5	NA	NA	NA	**79.4**	10.3

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configuration of C-6′ in the structures of **1** and **2** influences the antifungal activity of the parent compound. *R* configuration of C-6′ in structure **2** greatly decreased antifungal activity against *Colletotrichum* species, but slightly increased the antifungal activity against *Phomopsis* species. Comparing the structures of **1** and **3**, introduction of one hydroxyl group at the C-5′ position resulted in complete loss of the antifungal activity against *Colletotrichum* species and decreased the selectivity against *Phomopsis* species; this indicated the importance of maintaining an unsubstituted C-5′ for antifungal activity. By comparing structures **1** and **4**, the replacement of the hydroxyl group with the methoxy group at C-8 caused broad loss of the antifungal activity against all of the tested fungi, which indicated this position might be the active site where hydrogen bonds are formed. Comparison of compounds **3** and **5** revealed that the replacement of the hydrogen of the hydroxyl group at C-6 and the hydrogen at C-5′ with acetyl groups greatly increased the selectivity toward the two *Phomopsis* species.

In summary, fungi provide an abundant source of natural products that may have potential agricultural, environmental, and pharmaceutical use. In particular, *Cl. cladosporioides* provides a good source of natural cladosporin with a yield of 24% and should be considered as an important source of this metabolite by pharmaceutical and agrochemical companies. Compounds **1**, **2**, **3**, and **5** have potential utility as leads in the development of antifungal agrochemicals against certain plant pathogens. Compound **1** was tested to show specific antifungal, antibacterial, and antitumor properties, as well as insecticidal activity in previous studies.[16,20−23] Compound **1** was also reported to be a plant growth regulator (PGR), inhibiting etiolated wheat coleoptiles, but did not affect the growth of greenhouse-grown tobacco or corn.[24] Many commercial fungicides, such as the triazole class, show fungicidal and PGR activity and pharmaceutical applications. [25] However, commercial growers have learned to effectively use these compounds to their advantage and apply one chemical agent for both disease control and plant dwarfing. Fortunately, there are usually significant dose-dependent differences between fungicides and PGR effects in plants. However, overuse of this fungicide class can cause excessive dwarfing of some greenhouse crop plants (e.g., poinsettia). In the present study, compound **1** was evaluated for the first time against the filamentous fungal plant pathogens used in our microdilution broth assay and showed promising fungal growth inhibition. Moreover, the differences in activity indicated that the *S* configuration of C-6′, the openness of C-5′, the hydroxyl group at C-8, and the introduction of functional groups at C-6 influence the antifungal properties of these compounds. However, some literature papers have reported that *Cl. cladosporioides* could cause allergies and inflammation in sensitive patients at high concentration,[26−29] but they did not test cladosporin itself or the other metabolites. Therefore, further toxicity study on animals is needed for those metabolites to either prove or disprove this assumption.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

1D and 2D NMR spectra of the isolated compounds and details of the antifungal assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Smith, B. J. Anthracnose crown rot. In *Compendium of Strawberry Diseases*, 2nd ed.; Mass, J. L., Ed.; APS Press: St. Paul, MN, 1998; pp 46−48.

(2) Smith, B. J. Anthracnose fruit rot (black spot). In *Compendium of Strawberry Diseases*, 2nd ed.; Mass, J. L., Ed.; APS Press: St. Paul, MN, 1998; pp 31−33.

(3) Legard, D. E.; Whidden, A. J.; Chandler, C. K. Incidence and occurrence of strawberry diseases in Florida 1991−1996. *Adv. Strawberry Res.* **1997**, *16*, 35−47.

(4) Nita, M.; Ellis, M. A.; Madden, L. V. Effects of temperature, wetness duration and leaflet age on infection of strawberry foliage by *Phomopsis obscurans*. *Plant Dis.* **2003**, *87*, 579−584.

(5) Eshenaur, B. C.; Milholland, R. D. Factors influencing the growth of *Phomopsis obscurans* and disease development on strawberry leaf and runner tissue. *Plant Dis.* **1989**, *73*, 814−819.

(6) Wedge, D. E.; Nagle, D. G. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J. Nat. Prod.* **2000**, *63*, 1050−1054.

(7) Li, X.-J.; Zhang, Q.; Zhang, A.-L.; Gao, J.-M. Metabolites from *Aspergillus fumigatus*, an endophytic fungus associated with *Melia azedarach*, and their antifungal, antifeedant, and toxic activities. *J. Agric. Food Chem.* **2012**, *60*, 3424−3431.

(8) Bensch, K.; Groenewald, J. Z.; Dijksterhuis, J.; Starink-Willemse, M.; Andersen, B.; Summerell, B. A. H.-D.; Shin, H. D.; Dugan, F. M.; Schroers, H.-J.; Braun, U.; Crous, P. V. Species and ecological diversity within the *Cladosporium cladosporioides* complex (*Davidiellaceae*, *Capnodiales*). *Stud. Mycol.* **2010**, *67*, 1−94.

(9) Köhl, J. J.; Molhoek, W. W. M. L.; Haas, B. B. H. G.; Geijn, H. H. M. G. Selection and orchard testing of antagonists suppressing conidial production by the apple scab pathogen *Venturia inaequalis*. *Eur. J. Plant Pathol.* **2009**, *123*, 401−414.

(10) Jacyno, J. M.; Harwood, J. S.; Cutler, H. J.; Lee, M. K. Isocladosporin, a biologically ative isomer *Cladosporium cladosporioides*. *J. Nat. Prod.* **1993**, *56*, 1397−1401.

(11) Tabanca, N.; Bedir, E.; Slade, D.; Ferreira, D.; Wedge, D. E.; Jacob, M. R.; Khan, S. I.; Kirimer, N.; Baser, K. H. C.; Khan, I. A. Bioactive constituents from Turkish *Pimpinella* species. *Chem. Biodiversity* **2005**, *2*, 221−232.

(12) Meazza, G.; Dayan, F. E.; Wedge, D. E. Activity of quinones on *Colletotrichum* spp. *J. Agric. Food Chem.* **2003**, *51*, 3824−3828.

(13) Wedge, D. E.; Kuhajek, J. M. A microbioassay for fungicide discovery. *SAAS Bull. Biochem. Biotechnol.* **1998**, *11*, 1−7.

(14) Grove, J. F. New metabolic products of *Aspergillus flavus*. Part I. Asperentin its methyl ethers and 5-hydroxyasperentin. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2400−2406.
(15) Scott, P. M.; van Walbeek, W. Cladosporin a new antifungal metabolite from *Cladosporin cladosporioids*. *J. Antibiot.* **1971**, *24*, 747−755.
(16) Grove, J. F.; Pople, M. The insecticidal activity of some fungal dihydroisocoumarines. *Mycopathologia* **1981**, *76*, 65−67.
(17) Meazza, G.; Dayan, F. E.; Wedge, D. E. Activity of quinones on *Colletotrichum* species. *J. Agric. Food Chem.* **2003**, *51*, 3824−3828.
(18) Smith, B. J.; Wedge, D. E.; Pace, P. F. A microtiter assay shows effectiveness of fungicides for control of *Colletotrichum* spp. from strawberry. *Int. J. Fruit Sci.* **2013**, *13*, 205−216.
(19) Wang, S.; Li, X.-M.; Teuscher, F.; Li, D.-L.; Diesel, A.; Ebel, R.; Proksch, P.; Wang, B.-G. Chaetopyranin, a benzaldehyde derivative, and other related metabolites from *Chaetomium globosum*, an endophytic fungus derived from the marine red alga *Polysiphonia urceolata*. *J. Nat. Prod.* **2006**, *69*, 1622−1625.
(20) Podojil, M.; Sedmera, P.; Vokoun, J.; Betina, V.; Baráthová, H.; Ďuračková, Z.; Horáková, K.; Nemec, P. *Eurotium (Aspergillus) repens* metabolites and their biological activity. *Folia Microbiol.* **1979**, *23*, 438−443.
(21) Anke, H.; Zaehner, H.; Koenig, W. Metabolic products of microorganisms. 170. On the antibiotic activity of cladosporin. *Arch. Microbiol.* **1978**, *116*, 253−257.
(22) Xie, Q.-M.; Huang, J. -H. A preliminary study on Cladosporin. *J. Fujian Coll. For.* **1988**, *8*, 29−36.
(23) Bryant, F. O.; Cutler, H. G.; Parker, S. R.; Jacyno, J. M. Effect of fungal natural products in an *Agrobacterium tumefaciens* potato disc assay. *J. Nat. Prod.* **1994**, *57*, 640−643.
(24) Springer, J. P.; Cutler, H. G.; Crumley, F. G.; Cox, R. H.; Davis, E. E.; Thean, J. E. Plant growth regulatory effects and stereochemistry of cladosporin. *J. Agric. Food Chem.* **1981**, *29*, 853−855.
(25) Kocyigit-Kaymakcioglu, B.; Celen, A. O.; Tabanca, N.; Ali, A.; Khan, S. I.; Khan, I. A.; Wedge, D. E. Synthesis and biological activity of substituted urea and thiourea derivatives containing 1,2,4-triazole moieties. *Molecules* **2013**, *18*, 3562−3576.
(26) De Hoog, G. S.; Queiroz-Telles, F.; Haase, G.; Fernandez-Zeppenfeldt, G.; Attili Angelis, D.; Gerrits Van Den Ende, A. H.; Matos, T.; Peltroche-Llacsahuanga, H.; Pizzirani-Kleiner, A. A.; Rainer, J.; Richard-Yegres, N.; Vicente, V.; Yegres, F. Black fungi: clinical and pathogenic approaches. *Med. Mycol.* **2000**, *38* (Suppl. 1), 243−250.
(27) De Hoog, G. S.; Guarro, J.; Gené, J.; Figueras, M. J. In *Atlas of Clinical Fungi*,, 2nd ed.; ASM Press: Materials Park, OH, 2000.
(28) Vieira, M. R.; Milheiro, A.; Pacheco, F. A. Phaeohyphomycosis due to *Cladosporium cladosporioides*. *Med. Mycol.* **2001**, *39*, 135−137.
(29) Kwon-Chung, K. J.; Schwartz, I. S.; Rybak, B. J. A pulmonary fungus ball produced by *Cladosporium cladosporioides*. *Am. J. Clin. Pathol.* **1975**, *64*, 564−568.