Antifungal Metabolites Produced by *Chaetomium globosum* No.04, an Endophytic Fungus Isolated from *Ginkgo biloba*

Guizhen Zhang · Yanhua Zhang · Jianchun Qin ·
Xiaoyan Qu · Jinliang Liu · Xiang Li ·
Hongyu Pan

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Abstract The fungal endophyte *Chaetomium globosum* No.04 was isolated from the medicinal plant *Ginkgo biloba*. The crude extract of the fungus fermentation were active in the agar-diffusion tests against the phytopathogenic fungi *Rhizopus stolonifer* and *Coniothyrium diplodiella*. Further bioassay-guided chemical investigation led to the isolation and purification of six alkaloids and three non-targeted compounds from 50 L fermentation of this endophytic fungus and their structures were elucidated as chaetoglobosin A, C, D, E, G, R (**1–6**), ergosterol, allantoin and uracil, by means of spectroscopic analysis. Compounds **1–6** showed significant growth inhibitory activity against *R. stolonifer* and *C. diplodiella* at a concentration of 20 µg/disc. We present here, for the first time, the potent antifungal activity of chaetoglobosins from endophytic fungi against two important phytopathogenic fungi *R. stolonifer* and *C. diplodiella*.

Keywords *Chaetomium globosum* · Antifungal activity · Chaetoglobosins

Introduction

Rhizopus stolonifer and *Coniothyrium diplodiella* are phytopathogenic fungi that caused plant diseases such as peach rot disease and grape white rot. Over several decades, various methods had been used to prevent, control,

or eradicate plant diseases, and development of synthetic fungicides were primarily investigated [1, 2]. However, the global trend appears to be shifting towards reduced use of fungicides on produce and hence, there is a strong public and scientific desire to seek safer and eco-friendly alternatives to treat plant diseases in agriculture for reducing the decay loss [3]. Biological control provides an alternative to the use of synthetic fungicides with the advantages of greater public acceptance and reduced environmental impact [4].

Natural products have been a promising source of new lead compounds, and chemical agents in the agrochemical and pharmaceutical industries. Ever since the antibiotic drug penicillin was identified from *Penicillium* fungi in 1929, chemists have been focused interest on the discovery of novel bioactives from microbial metabolites. Despite engaged in a synthetic products, bioactive natural products retain a profound effect on modern medicine and agriculture.

Endophytes can be found in virtually all terrestrial plants and play important role for the growth of hosts [5]. Recently, endophytes have been recognized as rich sources of structurally unique and biologically active secondary metabolites [6–9].

During the course of our ongoing search for biologically active secondary metabolites from endophytic fungus [10–13], we investigated antifungal metabolites produced by cultures of the endophytic fungus *Chaetomium globosum* No.04, harbored inside the healthy barks of medicinal plant *Ginkgo biloba*, against phytopathogenic fungi *R. stolonifer* and *C. diplodiella* (Speq.) Sacc. Bioassay-guided fractionation of the crude extract of cultures of this fungus led to the isolation of six active cytochalasan type alkaloids along with three non-target metabolites. In the present paper, we report, for the first time, the potent antifungal activity of chaetoglobosins against these two agricultural

Guizhen Zhang and Yanhua Zhang contributed equally to this work and are considered co-first authors.

G. Zhang · Y. Zhang · J. Qin · X. Qu · J. Liu · X. Li ·
H. Pan (✉)
College of Plant Sciences, Changchun, Jilin, China
e-mail: panhongyu@jlu.edu.cn

important phytopathogenic fungi *R. stolonifer* and *C. diplodiella*, which can cause serious plant diseases.

Materials and Methods

Microorganisms and Culture Conditions

The fungal strain *C. globosum* No.04 was separated from the fresh barks of the host *Ginkgo biloba*, a medicinal plant growing in Linyi, Shandong province, China. It was authenticated by Prof. Hongyu Pan based on morphological studies and has been deposited at College of Plant Science, Jilin University. *R. stolonifer* and *C. diplodiella* were isolated from infectious peach and grape, respectively.

Fermentation

After growing on potato dextrose agar (PDA) medium at 25 °C for 4 days, the fresh mycelium of strain *C. globosum* No.04 was inoculated in liquid medium containing: Oat flour 80 g, maltose 10 g, yeast extracts 2 g dissolved in 1,000 mL dH₂O. The pH was adjusted to 6.0 before autoclaving. Fermentation was carried out in 2 L flasks each containing 1 L medium on a rotary shaker at 180 r/min, 25 °C for 8 days, and the cultures were used for the extraction and isolation.

General

Optical rotation was measured on a Perkin-Elmer 141 polarimeter. The melting point was determined on an XRC-1 micro-melting point apparatus and is uncorrected. IR spectra were obtained with a Nexus 870 FT-IR with KBr pellets. ¹H NMR spectra: Varian Inova 400 (399.95 MHz), 500 (499.8 MHz), 600 (600 MHz). ¹³C NMR spectra: Varian Inova 500 (125.7 MHz), 600 (150.9 MHz). Chemical shifts were measured relative to tetramethylsilane as an internal standard, δ were expressed in ppm. Mass spectra: EI-MS at 70 eV with Varian MAT 731, Varian 311A, AMD-402, high resolution with perfluorokerosine as standard. DCI-MS: Finnigan MAT 95 A, 200 eV, Reactant gas NH₃. Column chromatography was carried out on silica gel (200–300 mesh, Merck, German) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). All solvents used in this study were analytical grade. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10 % H₂SO₄ in ethanol.

Extraction and Isolation

The culture broth (50 L) of *C. globosum* No.04 was filtered to give the mycelium and water phase, The mycelium was dried at 45 °C, smashed and followed by ultrasonic extraction with

ethyl acetate and acetone three times successively to generated crude extract M (15.6 g). The culture filtrate was extracted by ethyl acetate three times and dried under vacuum to get crude extract F (20.5 g). Extracts M and F were combined due to both of them showed antifungal activity by agar-diffusion method. The total crude extract was chromatographed on a silica gel, eluted with a gradient system of CH₂Cl₂/MeOH (CH₂Cl₂, 50:1, 20:1, 10:1, MeOH) to give five fractions designated fractions A–E. Fraction B (8.5 g) was chromatographed over Sephadex LH-20 (CH₂Cl₂: MeOH = 6:4) and Sephadex LH-20 (MeOH) successively, one subfraction B-3-2 was repeatedly chromatographed over silica column (CH₂Cl₂:MeOH) and reversed-phase (ODS) column to afford the compound **1** (262.2 mg) and **2** (17.5 mg). Subfraction B-2-2 was repeatedly subjected to silica column (CH₂Cl₂: MeOH) to afford compound **5** (8.7 mg). Fraction C (7.2 g) was further separated by Sephadex LH-20 (CH₂Cl₂:MeOH = 6:4) and Sephadex LH-20 (MeOH) successively, and one subfraction C-4-2 was repeatedly chromatographed over silica column (CH₂Cl₂: MeOH) and reversed-phase (ODS) column to harvest compound **3** (6.8 mg) and **4** (6.0 mg). Subfraction C-5-2 was first fractionated on silica gel (CH₂Cl₂:MeOH), and then purified by reversed-phase (ODS) column to produce compound **6** (8.1 mg).

Antifungal Bioassay

For evaluation of the antagonistic potential of *C. globosum* No.04 against phytopathogenic fungi, we conducted the confrontation culture method [14, 16]. The *C. globosum* and phytopathogenic fungi were co-cultured on the 9-cm petri dish filled with PDA medium plate at 28 °C for 2–4 days to check the confrontation effect between them. The distance between the two inoculation sites (strain No.04 and fungal plug) on each plate was 45 mm.

The antifungal effect of the extract, fraction and active compounds was tested by Bauer-Kirby method (agar-diffusion method) [15] with 6-mm diameter filter paper discs. The paper disc was impregnated with 4 μ L of dimethyl sulfoxide (DMSO) solution of 5 mg/mL compounds 1–6 (20 μ g/disc) and the discs for negative control was treated with 4 μ L of DMSO. Hygromycin B (25 mg/mL) was used as positive drug, (the paper discs were treated with same amounts of DMSO and dried). After the 6-mm diameter filter papers were completely dry on both sides, they were placed on the side margins of the assay PDA plates, tested phytopathogenic fungi were inoculated in the center of plates, The distance between the two inoculation sites (each filter paper and fungal plug) on each plate was about 35 mm. The inhibition zones around each disc of *R. stolonifer* and *C. diplodiella* were measured when the after 24 and 72 h respectively. Each treatment contains triplicate petri dishes and the experiment was repeated three times.

Fig. 1 Influence of *Chaetomium globosum* No.04 (A) on *Rhizopus stolonifer* (B, left) and *Coniothyrium diplodiella* (B, right) by confrontation culture method

Evaluation of the Antifungal Activity of Chaetoglobosin A

The antifungal activities of chaetoglobosin A against *R. stolonifer* and *C. diplodiella* were determined on solid PDA mediums. The freshly prepared and sterilized PDA medium (45 °C) was mixed with chaetoglobosin A (5 mg/mL) for the final concentrations of 0.015, 0.030, 0.0625, 0.125, 0.250, 0.500, 1.000, 2.000 µg/mL and then evenly distributed to several Petri dishes. PDA Petri dishes which contain little DMSO but without chaetoglobosin A was used as negative control. Then three mycelia discs from 3-day-old fungal cultures were cultured in an equal space in the Petri dishes and incubated at 26 °C. The mycelia diameters of phytopathogenic fungi were recorded after 24–72 h culturing and the inhibitive percentage was calculated by the following formula: Inhibition percent = $x = \frac{(d_t - d_c)}{(d_t - d_i)} \times 100 \%$. Where d_c is the mean colony diameter (or radius) of negative control sets; d_t is the mean colony diameter (or radius) of treatment sets; d_i is the initial colony diameter (or radius) of fungal PDA discs. For each treatment, three replicates were performed. The minimal inhibitory concentrations (MICs) were recorded by reading the lowest concentrations that inhibited visible growth. Effective concentrations for inhibition of 50 and 90 % of fungi radial growth (EC_{50} and EC_{90}) were calculated by the program DPS.

Data Analysis

Data were statistically processed and subjected to analysis of variance (ANOVA) using the program DPS and means

significantly different were separated by the least significant difference (LSD) with $p < 0.05$.

Results and Discussion

Isolation and Identification of Antifungal Metabolites

Primary screening for the antagonism of the endophytic fungus by confrontation assay [14–16] was performed against phytopathogenic fungi *R. stolonifer* and *C. diplodiella* (Speq.) Sacc. (Fig. 1). Activity-directed fractionation of the crude extract of cultures of this fungus led to the isolation of six active cytochalasan type alkaloids along with three non-target metabolites. Their structures were determined as chaetoglobosin A (1), C (2), D (3), E (4), G (5), R (6) (Fig. 2), ergosterol, allantoin and uracil on the basis of ESI-MS, ^1H and ^{13}C NMR together with 2D-NMR spectroscopic analysis, as well as comparison of chemical-physic properties with those of previously reported data in the literatures [17–22]. In the present paper, we report, for the first time, the potent antifungal activity of chaetoglobosins against these two agricultural important phytopathogenic fungi *R. stolonifer* and *C. diplodiella*, which can cause serious plant diseases, such as peach rhizopu rot and grape white rot, respectively.

Chaetoglobosins belong to the general group of cytochalasans with the phenyl group in the molecules being replaced by an indolyl group [22], and to date around 40 chaetoglobosins and analogues have been characterized from cultures of the fungus genus *Chaetomium*, *Iplodia*, *Calonectria*, *Cylindrocladium*, *Discosia*, *Penicillium* and *Phomopsis* [23,

Fig. 2 Structures of compounds **1–6** **Fig. 3** Percentage inhibition of phytopathogenic fungi (*Rhizopus stolonifer* and *Coniothyrium diplodiella*) at compounds **1–6**. Each point is the mean of three independent experiments. Error bars denote SE

[24], which includes both marine- and terrestrial-derived species. However, *Chaetomium* species was revealed to be an excellent rich source of chaetoglobosins [25].

In Vitro Antifungal Activity of Isolated Compounds

In vitro antifungal effects of compounds **1–6** were evaluated by agar-diffusion method [15]. Compounds **1–6** show

Table 1 Bioactivity of chaetoglobosin A against phytopathogenic fungi

Phytopathogenic fungi	MIC (μg/mL)	EC ₅₀ (μg/mL)	EC ₉₀ (μg/mL)
<i>Coniothyrium diplodiella</i>	<0.0150	0.1073 ± 0.0164 ^a	0.5800 ± 0.0232 ^a
<i>Rhizopus stolonifer</i>	<0.0625	0.6663 ± 0.0465 ^b	1.9862 ± 0.0580 ^b

Each point is the mean of three independent experiments. Means (±SE) in the same column followed by different letters (^a and ^b) within the same column indicated differ significantly ($p < 0.05$) according to the Fisher's protected LSD test

moderate antifungal effects against *R. stolonifer* with observed acute inhibition percent range from 31.37–51.53 %, and display remarkable inhibitory effects against *C. diplodiella* with average inhibition percent in the range 54.73–73.07 % (Fig. 3), at a concentration of 20 μg/disc. The average inhibition zone radius of positive control hygromycin B (100 μg/disc) against *R. stolonifer* and *C. diplodiella* was 4.5 and 32.5 mm, respectively. There was no clear sterile zone was observed around negative control.

Reportedly, chaetoglobosins, such as chaetoglobosin A (**1**), were known as cytochalasan alkaloids exhibits strong cytotoxicity on various of human cancer cell lines [17, 23].

Fig. 4 **a** Linear regressions of colonies inhibition percent of *Coniothyrium diplodiella* at different concentrations of chaetoglobosin A (0.015, 0.030, 0.0625, 0.125, 0.250, 0.500 µg/mL). **b** Linear regressions of colonies inhibition percent of *Rhizopus stolonifer* at different concentrations of chaetoglobosin A (0.0625, 0.125, 0.250, 0.500, 1.000, 2.000 µg/mL)

Chaetoglobosin A (**1**), **C** (**2**), **D** (**3**), **E** (**4**) and **G** (**5**) displayed acute toxic effects against Hela cells with IC_{50} values in the range 3.2–20 µM [21]. Compounds **1**, **3** and **6** possess significant toxicity against P388 murine leukemia cell line [22]. Compounds **2**, **3** and **5** showed cytotoxicities against BC1 cell lines, KKU-100 cells and KKU-OCA17 cells with IC_{50} values in the range 2.54–21.29 µM [26]. Compounds **1** and **2** showed significant growth inhibitory activity against the brine shrimp (*Artemia salina*) [10], also exhibited highly toxic effects to mice and rats cells and chick embryos [28, 29]. **1** and **2** also showed phytotoxicity against alfalfa seedlings, antibacterial activity against *Helicobacter pylori* and *Staphylococcus aureus* [22, 27], as well as marked inhibitory effects on the fungus *Mucor miehei* in an agar-diffusion assay [10].

Antifungal Activity of Chaetoglobosin A on Phytopathogenic Fungi

Antifungal activities of different concentrations of chaetoglobosin A on phytopathogenic fungi were evaluated. As shown in Table 1, the mycelia growths were inhibited in the presence of chaetoglobosin A, and the inhibitory efficiency were enhanced with the increment of the concentration of chaetoglobosin A. The mycelia growths of 24 h cultivation of *R. stolonifer* and 72 h cultivation of *C. diplodiella* were nearly completely inhibited as chaetoglobosin A concentration 40 and 20 µg/mL, respectively. Linear regressions of colonies inhibition percent of *R. stolonifer* and *C. diplodiella* by different concentrations of chaetoglobosin A shown in (Fig. 4). As the MIC, EC_{50} and EC_{90} of chaetoglobosin A against *R. stolonifer* and *C. diplodiella* shown in (Table 1), chaetoglobosin A had acute antifungal activity against phytopathogenic fungi, which indicated that the studied propolis had the potential to be a natural preservative that can be applied to control plant diseases.

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