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Full Length Research Paper

Antifungal metabolites from *Phomopsis* sp. By254, an endophytic fungus in *Gossypium hirsutum*

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Chemical and bioassay-guided fractionation of the chloroform-methanol (1:1) extract of *Phomopsis* sp. By254, an endophytic fungus residing in the root of *Gossypium hirsutum*, yielded three compounds. The structures of these compounds were elucidated by a combination of spectroscopic analysis (MS, 1D- and 2D-NMR) to be epoxycytochalasin H (1), cytochalasin N (2), and cytochalasin H (3). All the compounds were subjected to antimicrobial action against plant pathogenic fungi and plant pathogenic bacteria. As a result, compounds 1 to 3 significantly inhibited the growth of *Sclerotinia sclerotiorum*, *Bipolaris maydis*, *Fusarium oxysporum*, *Botrytis cinerea, Bipolaris sorokiniana*, *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia cerealis*, with IC_{50} value ranging from 0.1 to 50 µg/ml. However, no inhibition of them was observed against plant pathogenic bacteria.

Key words: *Phomopsis* sp., *Gossypium hirsutum*, metabolites, antifungal, antibacterial.

INTRODUCTION

Agricultural plant diseases from the plant pathogenic fungi and bacteria, are one of the major economic damages in agriculture in the world, which cause harvest losses in crop production may amount to 12% or even higher in developing countries (Jin et al., 2009). Although these losses may be attenuated by the use of crop rotation, sanitation practices or resistant cultivars, fungicides are often also needed to maximize crop yields (Russell, 2005). However, strategies to control plant diseases with classic fungicides may produce harmful side effects, such as serious environmental pollution and the development of multiresistant strains. Thus, there is a continuing need for more effective and safer fungicides, especially those with novel modes of action, and natural products play a key role in the search for such compounds (Liu et al., 2007; Wegulo et al., 2011). Endophytes, living for most of their life cycles inside healthy plant tissues, have been proven to be a rich and reliable source of biologically active and/or chemically novel compounds for exploitation in modern medicine, agriculture and industry (Yu et al., 2010; Schulz and Boyle, 2005). As one class of the most widely distributed endophytic fungi, *Phomopsis* sp. have attracted much attention in recent years for their ability to produce a variety of bioactive secondary metabolites (Vatcharin et al., 2008; Yang et al., 2010). The cytochalasins are a class of fungal secondary metabolites, which inhibit a variety of cellular movements including cell division and motility, and cause changes in cell shape (Zhang et al., 2008). Cytochalasins are cytotoxic mycotoxins that act on the cytoskeleton but may show various other bioactivities (Cole and Schweikert, 2003). For instance, biological assays indicated that cytochalasin Z17 had moderate cytotoxicity against human nasopharyngeal epidermoid tumor KB cell line (Zhang et al., 2010), and alachalasins D and G showed modest antimicrobial activity (Zhang et al., 2008). Cytochalasin L-696, 474 from *Hypoxylon fragiforme* strongly inhibited human immunodeficiency virus (HIV)-1 protease (Lingham et al., 1992). A significant inhibitory activity on *Phytophthora cactorum* was shown by cytochalasin B at $2 \times 10^{-5} – 2 \times 10^{-4}$ M

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(Evidente et al., 1997).

In our previous screening for biologically active metabolites from endophytic microorganisms, total 103 strains were isolated from the endorhiza of field-grown cotton plants (Zheng et al., 2011). Among them, the culture extract of endophytic fungus *Phomopsis* sp. By254 displayed strong antagonistic activity to a number of phytopathogenic fungi. The aim of the present work was to isolate and identify bioactive secondary metabolites from this fungus. As a result the antifungal compounds produced by strain By254 were isolated and characterized to be members of the cytochalasin family.

MATERIALS AND METHODS

General

NMR spectra were acquired on a Bruker DRX-500 NMR spectrometer using TMS and solvent signals as internal standards. ESI-MS were taken on a Mariner Mass 5304 instrument. Silica gel (200 to 300 mesh) for column chromatography and silica GF_{254} for TLC were produced by Qingdao Marine Chemical Company, China. Chemical samples were prepared on a Shimadzu LC20AT HPLC system with ODS column (250 L × 4.6,shimadzu pak) at a flow rate of 1.0 ml/min.

Microorganisms and fermentation

The strain By254 was isolated from the root of *Gossypium hirsutum*, collected in 2006 from the suburb of Nantong, China, according to the procedure described elsewhere (Miruna et al., 2006). Specifically, the root of *G. hirsutum* was washed with running tap water, sterilized with 70% ethanol for 30 s and 1% sodium hypochlorite for 1 min, then rinsed in sterile water for three times and placed in Martin agar medium (10 g glucose, 5 g peptone, 1 g KH_2PO4, 3.3 ml 1% (w/v) Rose Bengal solution, 0.5 g $MgSO_4$ $7H_2O$, 20 g agar, and 1000 ml distilled water) (Zheng et al., 2011), supplemented with streptomycin and cultivated at 25℃ according to the method described by Qu et al. (2005). The isolated endophytic fungus was preserved on potato-dextrose-agar (PDA) slants at 4℃. The endophytic fungus was identified according to colony morphology and conidial size of cultures, which was reinforced by the internal transcribed spacer (ITS) region (White et al., 1990) that gave a 99% sequence similarity to those accessible at the BLAST of *Phompsis* sp. (GenBank accession number: GQ365159). The strain By254 was cultured by a solid-matrix steady protocol (Liu et al., 2004). Briefly, the fresh mycelium grown on PDA medium at 28℃ for 5 days was inoculated into 1000 ml Erlenmeyer flasks containing 400 ml PD medium.

After 6 days of incubation at 28℃ on rotary shaker at 150 rpm, 20 ml of culture liquid was transferred as seed into 300 bottles preloaded with a given amount of grain medium composed of 7.5 g millet, 7.5 g bran, 0.5 g yeast extract, 0.1 g tartrate sodium, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.1 g glutamine sodium, 0.1 ml pure corn oil and 30 ml water. Cultivation was kept at 28 ± 1℃ with 60 to 70% humidity for 35 days.

Extraction and isolation of secondary metabolites

The biomass of the fungal strain (1.5 kg, incompletely dried) was collected after cultivation on solid medium for 35 days, extracted with chloroform-methanol (1:1) (4 × 5 L) for four times and concentrated *in vacuo* to give a brown residue (250 g). After eliminating waxy substances, a residue (165 g) was obtained, which was then chromatographed over a silica gel column (1000 g, 200 to 300 mesh) eluted successively with $CHCl_3$/MeOH gradient (100:0, 100:1, 100:2, 100:4, 100:8, 100:16, 100:50, 0:100, v/v) to obtain eight fractions (Fr1, Fr2, Fr3, Fr4, Fr5, Fr6, Fr7, Fr8). The antifungal activity of each fraction against *Pythium ultimum*, *Fusarium graminearum* and *Sclerotinia sclerotiorum* were tested by a modified paper-disk method (Taechowisan et al., 2005). The bioactive Fr3 (14.1 g) was subjected to further column chromatography fractionation over silica gel (200 g) with $CHCl_3$/MeOH gradient, with the active part (0.56 g) separated further by silica gel (20 g) with $CHCl_3$/MeOH gradient to give compound 1 (0.24 g). The bioactive Fr4 (1.93 g) was further fractionationed over silica gel (9.0 g) with $CHCl_3$/MeOH gradient, with the active part (0.8 g) separated by silica gel (9.0 g) with $CHCl_3$/MeOH gradient, and was further purified by high performance liquid chromatography (HPLC) using MeOH/H_2O (65:35) (1.0 ml/min) to yield compound 2 (0.44 g, t_R = 16.4 min) and compound 3 (0.12 g, t_R = 10.8 min).

Antimicrobial activities tests

Antifungal tests were done in quadruplet with *P. ultimum*, *F. graminearum*, *S. sclerotiorum*, *Bipolaris maydis*, *Fusarium. oxysporum*, *Phytopathora capsici*, *Botrytis cinerea*, *Bipolaris sorokiniana*, *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis* by a modified paper-disk method (Taechowisan et al., 2005). Briefly, plates were inoculated with agar discs of fungal strains in the center of the plate and with paper discs for addition of metabolites on the side. Whatmann filter paper discs of 8 mm diameter were impregnated with 10 µl of each isolated compound at a concentration of 1 µg/µl (10 µg/disc) prepared using acetone. The discs were evaporated *in vacuo* and were placed at 1 cm from each of the edges of the plate and were diagonally separated by a distance of 7 cm. All operations were performed under sterile conditions. Then, plates were incubated at 25℃. The inhibitory activity was estimated from measurements of fungal growth across the line separating the discs compared to a control in which only acetone was added. Antibacterial tests were done in quadruplet with *Curtobacterium luteum* (ATCC15830), *curtobacterium. flaccumfaciens* pv. *flaccumfaciens* (NZ2580), *Acidovorax aveane* subsp. *citrulli* (FC440), *Clavibacter michiganensis* subsp. *sepedonicus* (9667), *C. michiganensis* subsp. *michiganensis* (ATCC10202), *C. michiganensis* subsp. *nebraskensis* (ATCC27822), *Xanthomonas campestris* pv. *campestris* (XCC), *X. campestris* pv. *oryzae*, *Rhodococcus fascians* (NZ5833) and *Ralstonia solanacearum* (ZJ6072103) by disk diffusion method (Murray et al., 1995). The inocula of microorganisms were prepared from 12 to 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Then 100 µl of bacterial suspension were spread on nutrient agar (NA), respectively.

The disks (8 mm in diameter), containing 10 µl of each isolated compound at a concentration of 1 µg/µl (10 µg/disk), were impregnated in the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the isolated compounds. Tetracycline was used as positive reference standard to determine the sensitivity of isolated compounds for each microbial species tested. The inoculated plates were incubated at 30℃ for 24 h. Antibacterial activity was evaluated by measuring the inhibition zones in reference to the test organisms.

Half maximal inhibitory concentration (IC_{50}) tests

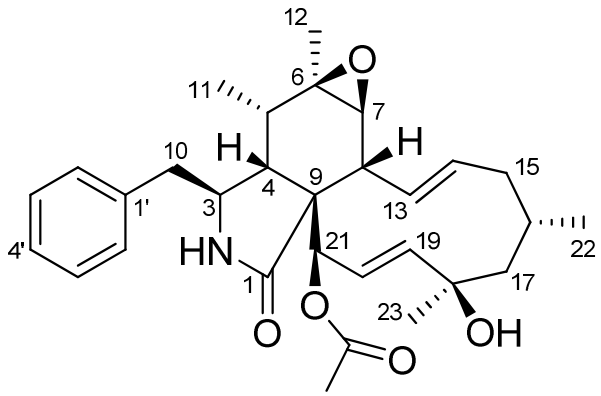
Purified compounds were assayed on PDA in Petri dishes to determine the IC_{50} of these compounds against nine plant

pathogenic fungi (Elson et al., 1994). A stock solution (40 µg/µl) of the purified compound was prepared, which was further diluted serially with acetone or DMSO. These solutions were added to PDA media contained in conical flasks at 48°C to obtain the final concentrations at 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. The medium (5 ml) was added to a 5 cm diameter Petri dish. A 6 mm diameter plug of the fungi, removed from the margin of a 4-day-old colony on PDA, was placed in the center of the plate. Linear growth of the fungi at 25°C was recorded till the fungal growth was almost complete in the control plates. Each treatment consisted of three replicates. IC_{50} value was defined as the concentration required for 50% inhibition of microbial growth.

RESULTS AND DISCUSSION

The organic extract derived from the solid-substrate fermentation materials of the endophytic fungus *Phomopsis* sp. By254 was chromatographed repeatedly on silica gel and HPLC to yield three compounds (Figure 1) with the same molecular weight [ESI-MS m/z: 516.3$(M + Na)^+$, 1010.7$(2 M + Na)^+$] at 493.3, which were identified as epoxycytochalasin H (1), cytochalasin N (2) and cytochalasin H (3), respectively, according to their spectral and physical data (Table 1). The ^1H- and ^{13}C - NMR data are consistent with the literature reported (Izawa et al., 1989). All three compounds were tested *in vitro* for the antifungal activity against tested pathogenic fungi, and the results were listed in Table 2. The growths of nine plant pathogenic fungi were inhibited by compounds 1 to 3 except *P. capsici*. The result of IC_{50} tests (Table 3) showed that compounds 1 to 3 completely inhibited *S. sclerotiorum*, *B. maydis*, *F. oxysporum*, *B. cinerea, B. sorokiniana*, *G. graminis* var. *tritici* and *R. cerealis*, with IC_{50} value ranging from 0.1 to 50 µg/ml. Compounds 1 to 3 showed growth inhibition against *B. maydis* with IC_{50} value of 1.87, 1.91, 1.18 µg/ml, respectively. Compound 3 inhibited the growth of *G. graminis* var. *tritici* with IC_{50} value of 0.15. The IC_{50} value of cycloheximide used as a positive reference in the study against *B. maydis* and *G. graminis* var. *tritici* were 9.71 and 0.37. However, no inhibition of them was observed against plant pathogenic bacteria in the antibacterial tests. Compounds 1 to 3 had been isolated from *Phomopsis* sp. 68-GO-164 (Izawa et al., 1989). It was reported that compounds 1 and 3 were also produced by *Pestalotia* sp., which is plant - associated imperfect fungi belonging to the same class Coelomycetes as *Phomopsis* sp. (Suttun, 1980).

Extensive research had been done with their cytotoxicity. Compound 3 exhibited strong cytotoxicity toward KB cells and KBv200 cells with IC_{50} less than 1.25 µg/mL (Tao et al., 2008), but had no effect on HIV-1 protease activity (Lingham et al., 1992). Compounds 1 and 3 inhibited the murine mixed lymphocyte reaction (MLR) with IC_{50} value of less than 10 µg/ml (Burres et al., 1992). However, little is known about their antimicrobial activity. *In vitro*, compound 3 exhibited antibacterial, antifungal, nematocidal and antitumor activity. Compound 3 from *H. fragiforme* and its derivatives showed antimicrobial activities against *Bacillus subtilis*, *Yarrowia lipolytica*, and various filamentous fungi including *Mucor hiemalis*, *Penicillium griseofulvum*, *Stachybotrys*



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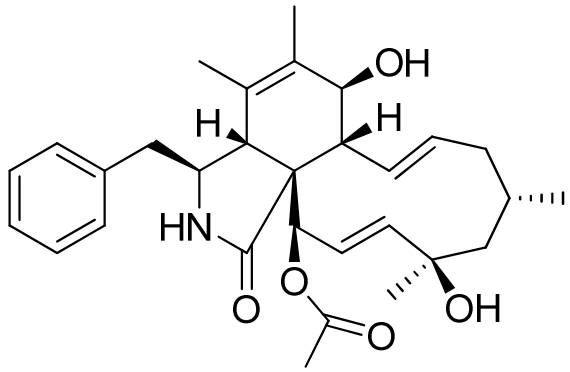

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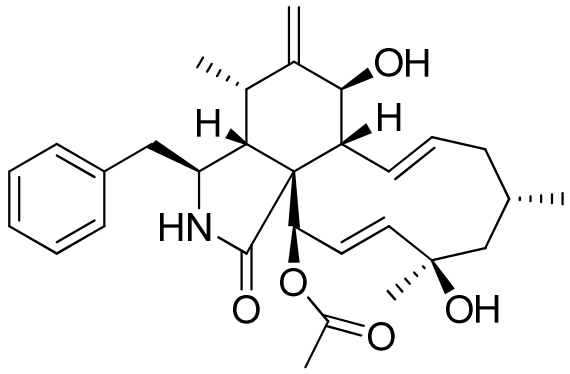

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Figure 1. Structure of the compounds 1 to 3 isolated from *Phomopsis* sp. By254.

Table 1. ^{1}H- and ^{13}C-NMR data for compounds 1 to 3 (500MHz, CD_3OD).

No.	1		2		3	
	δ_C (ppm)	δ_H (*J* in Hz)	δ_C (ppm)	δ_H (*J* in Hz)	δ_C (ppm)	δ_H (*J* in Hz)
1	175.2		175.0		174.3	
2	NH		NH		NH	
3	54.2	3.60(1H, *m*)	60.4	3.34(1H, *m*)	53.7	3.24(1H, *m*)
4	50.9	2.08(1H, *m*)	49.9	2.47(1H, *br s*)	50.2	2.12(1H, *dd,* 4.1)
5	36.7	1.70(1H, *m*)	126.2		32.8	2.77(1H, *m*)
6	57.0		131.8		148.0	
7	62.9	2.75(1H, *d,* 5.7)	68.2	3.8(1H, *d,* 9.4 Hz)	69.7	3.82(1H, *d,* 10.8)
8	45.2	2.62(1H*, dd,* 9.7, 5.7)	50.3	2.53(1H, *dd,* 10.0, 10.0)	47.1	2.93(1H, *dd,* 10.2, 10.2)
9	53.5		51.1		51.7	
10	46.0	2.78(1H, *d*, 5.7)	44.9	2.97(2H, *m*)	45.46	2.85(1H, *dd*, 13.4, 4.6)
		2.80(1H, *d*, 7.2)				2.66(1H, *dd*, 13.3, 9.5)
11	12.6	0.86(3H, *d,* 7.3)	17.2	1.44(3H, *s*)	13.95	0.97(3H, *d*, 6.6)
12	19.6	1.20(3H, *s*)	13.9	1.71(3H, *s*)	113.96	5.10(1H, *s*)
						5.33(1H, *s*)
13	127.7	5.87(1H, *dd*, 15.5, 9.9)	127.8	5.99(1H,*dd*, 15.8, 10.2)	125.8	5.84(1H, *dd,* 15.5, 9.8)
14	135.8	5.30(1H, *m*)	138.3	5.41(1H, *m*)	138.1	5.40(1H, *m*)
15	42.8	1.77(1H, *m*)	42.8	1.83(1H, *m*)	42.7	1.8(1H, *m*)
		2.02(1H, *dd*, 10.3, 5.1)		2.02(1H, *dd*, 12.2, 5.3)		2.0(1H, *m*)
16	28.2	1.79(1H, *m*)	28.3	1.79(1H, *m*)	31.0	1.33(1H, *s*)
17	53.7	1.86(1H, *m*)	53.9	1.88(1H, *dd,* 14.5, 3.1)	53.6	1.56(1H, *d,* 14.5)
		1.57(1H, *m*)		1.58(1H, *dd,* 14.5, 2.9)		1.86(1H, *dd,* 14.5, 2.0)
18	74.3		74.4		74.2	
19	125.4	5.79(1H, *dd*, 16.5, 2.3)	138.9	5.55(1H, *dd*, 16.4, 2.0)	127.0	5.84(1H, *dd*, 17.2, 1.5)
20	138.1	5.49(1H, *dd,*16.5, 2.2)	125.5	5.58(1H, *d*, 2.0)	138.5	5.54(1H, *br s*)
21	76.1	5.69(1H, *br s*)	75.3	5.78(1H, *t*, 2.1)	77.4	5.51(1H, *d*, 14.3)
22	26.3	1.04(3H, *d,* 5.9)	26.4	1.05(3H, *d*, 6.6)	26.4	1.04(3H, *d*, 6.0)
23	30.7	1.34(3H, *s*)	30.6	1.37(3H, *s*)	28.3	1.82(3H, *s*)
21-Ac	170.2		170.4		170.1	
	20.8	2.21(3H, *s*)	20.9	2.25(3H, *s*)	20.78	2.24(3H, *s*)
1’	137.0		137.6		137.3	
2’, 6’	129.1	7.17(2H, *d*, 7.3)	129.1	7.14(2H, *d*, 7.2)	128.99	7.14(2H, *d,* 7.3)
3’, 5’	128.9	7.32(2H, *dd*, 14.7, 7.3)	128.9	7.32(2H, *dd*, 14.8, 2.3)	128.8	7.32(2H, *dd,* 7.3)
4’	127.1	7.26(1H, *t*, 7.2)	127.0	7.25(1H, *t,* 8.2)	127.0	7.25(1H, *t,* 7.3)

Abbreviations of signal multiplicity: *br s*, broad singlet; *d*, doublet; *dd*, doublet of doublets; *ddd*, doublet of double doublets; *m*, multiplet; *s*, singlet; *t*, triplet.

chartarum, *Trichoderma atroviride*, *Trichoderma. harziamum* (Stadler et al., 2006). To our knowledge, this is the first report to demonstrate antifungal activities of compounds 1 to 3 against tested fungi. In most cases, antibiotics showing *in vitro* activity are generally active *in vivo* against plant diseases. The evaluation of *in vitro* antifungal activity of an antibiotic is the prerequisite for *in vivo* evaluation of its antifungal activity. Further evaluation of the effectiveness of compounds 1 to 3 for disease control and applications should be done in the field.

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Table 2. Radial growth inhibition of compounds 1 to 3 against plant pathogenic fungi.

Microorganisms	Inhibition zone in radius (mm) around test disk			
	1*	2*	3*	Cycloheximide*
Pythium ultimum	+	+ +	+ +	+
Sclerotinia sclerotiorum	+ +	+ +	+ +	+ + +
Fusarium graminearum	+ +	+	+	+ +
Rhizoctonia cerealis	+ +	+ +	+ + +	+ + +
Gaeumannomyces graminis var. *tritici*	+ + +	+ +	+ + +	+ + +
Bipolaris maydis	+ + +	+ + +	+ + +	+ +
Bipolaris sorokiniana	+ +	+	+ +	+
Botrytis cinerea	+ +	+ +	+ +	+
Fusarium oxysporum	+ +	+	+	+ +

Inhibition zone in radius was reported as "+" 0-1.0mm, "+ +" 1.0-5.0mm, "+ + +" 5.0-10.0mm. * At the amount of 10 µg compound on each paper disk.

Table 3. IC_{50} of compounds 1 to 3 against plant pathogenic fungi.

Microorganisms	IC_{50} (µg/mL)#			
	1	2	3	Cycloheximide
Pythium ultimum	>100	24.26±0.07	3.52±0.03	75.93±0.02
Sclerotinia sclerotiorum	8.21±0.03	2.45±0.03	8.65±0.03	6.12±0.02
Fusarium graminearum	39.40±0.03	>100	57.35±0.04	15.54±0.01
Rhizoctonia cerealis	8.63±0.02	11.12±0.02	8.92±0.02	1.25±0.03
Gaeumannomyces graminis var tritici	5.73±0.02	13.04±0.03	0.12±0.02	0.36±0.01
Bipolaris maydis	1.87±0.01	1.89±0.03	1.16±0.01	9.71±0.01
Bipolaris sorokiniana	6.94±0.01	48.81±0.04	19.91±0.03	>100
Botrytis cinerea	6.36±0.02	5.62±0.02	5.57±0.01	>100
Fusarium oxysporum	11.80±0.01	25.89±0.03	23.81±0.04	17.46±0.01

#The concentration of compound required for 50% inhibition of microbial growth.

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REFERENCES

Burres NS, Premachandran U, Humphrey PE, Jackson M, Chen RH (1992). A new immunosuppressive cytochalasin isolated from a *Pestalotia* sp. J. Antibiot., 45: 1367-1369.

Changes in secondary metabolism during stromatal ontogeny of *Hypoxylon fragiforme*. Mycol. Res., 110: 811-820.

Cole RJ, Schweikert MA (2003). Handbook of secondary fungal metabolites. Academic Press, New York. Vol. 1.

Elson MK, Kelly JF, Nair MG (1994). Influence of antifungal compounds from a soil-borne actinomycete on *Fusarium* spp. in asparagus. J. Chem. Ecol., 20: 2835-2846.

Evidente A, Cristinzio G, Capasson R, Andolfi A (1997). Fungitoxic activity of some cytochalasins and their derivatives on *Phytophthora* species. Nat. Toxins., 5: 228-233.

Izawa Y, Hirose T, Shimizu T, Koyama K, Natori S (1989). Six new 10-phenyl-[11]cytochalasans, cytochalasins N-S from *Phomopsis* sp. Tetrahedron., 45(8): 2323-2335.

Jin H, Geng YC, Yu ZY, Tao K, Hou TP (2009). Lead optimization and anti-plant pathogenic fungi activities of daphneolone analogues from *Stellera chamaejasme* L. Pestic. Biochem. Phys., 93: 133-137.

Lingham RB, Hsu A, Silverman KC, Bills GF, Dombrowski A, Goldman ME, Darke PL, Huang L, Koch G (1992). L-696,474, a novel cytochalasin as an inhibitor of HIV-1 protease. III. Biological activity. J. Antibiot., 45: 686–691.

Liu CH, Chen X, Liu TT, Lian B, Gu YC, Caer V, Xue YR, Wang BT (2007). Study of the antifungal activity of *Acinetobacter baumannii* LCH001 *in vitro* and identification of its antifungal components. Appl. Microbiol. Biotechnol., 76: 459-466.

Liu JY, Song YC, Zhang Z, Wang L, Guo ZJ, Zou WX, Tan RX (2004). *Aspergillus fumigatus* CY018, an endophytic fungus in *Cynodon dactylon* as a versatile producer of new and bioactive metabolites. J. Biotechnol., 114: 279-287.

Miruna OS, Gomes Newton CM, Neuber G, Smalla K (2006). A new semi-nested PCR protocol to amplify large 18S *rRNA* gene fragments for PCR-DGGE analysis of soil fungal communities. J. Microbiol. Meth., 65: 63-75.

Murray PR, Baron EJ, Pfaller MA (1995). Manual of Clinical Microbiology, 6th ed. ASM, Washington DC., 6: 214-221.

Qu ZL, Wang HY, Xia GX (2005). GhHb1: a nonsymbiotic hemoglobin gene of cotton responsive to infection by *Verticillium dahliae*. Biochim. Biophys. Acta., 1730: 103-113.

Russell PE (2005). A century of fungicide evolution. J. Agric. Sci., 143: 11-25.

Schulz B, Boyle C (2005). The endophytic continuum. Mycol. Res., 109: 661–686.

Stadler M, Quang DN, Tomita A, Hashimoto T, Asakawa Y (2006).

Suttun BC (1980). The Ceolomycetes. Commonwealth Mycological Institute.

Taechowisan T, Lu CH, Shen YM, Lumyong S (2005). Secondary metabolites from endophytic *Streptomyces aureofaciens* CMUAc130 and their antifungal activity. Microbiol., 151: 1691-1695.

Tao YW, Zeng XJ, Mou CB, Li J, Cai XL, She ZG, Zhou SN, Lin YC (2008). ^{1}H and ^{13}C NMR assignments of three nitrogen containing compounds from the mangrove endophytic fungus (ZZF08). Magn. Reson. Chem., 46: 501-505.

Vatcharin R, Ubonta S, Souwalak P, Jariya S (2008). Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. Phytochemistry., 69: 783-787.

Wegulo SN, Zwingman MV, Breathnach JA, Baenziger SP(2011). Economic returns from fungicide application to control foliar fungal diseases in winter wheat. Crop Prot., 30: 685-692.

White TJ, Bruns T, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In. Innis MA, Gelfand DH,. Sninsky JJ, White TJ (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York, pp. 315-322.

Yang JX, Xu F, Huang CH, Li J, She ZG, Pei Z, Lin YC (2010). Metabolites from the mangrove endophytic fungus *Phomopsis* sp (#zsu-H76). Eur. J. Organ. Chem., 19: 3692-3695.

Yu HS, Zhang L, Li L, Zheng CJ, Guo L, Li WC (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol. Res., 165: 437-449.

Zhang HW, Zhang J, Hu S, Zhang ZJ, Zhu CJ, Ng SW, Tan RX (2010). Ardeemins and cytochalasins from *Aspergillus terreus* residing in *Artemisia annua*. Planta Med., 76: 1616-1621.

Zhang YG, Tian RR, Liu SC, Chen XL, Liu XZ, Che YS (2008). Alachalasins A-G, new cytochalasins from the fungus *Stachybotrys charatum*. Bioorgan. Med. Chem., 16: 2627-2634.

Zheng Y, Xue QY, Xu LL, Xu Q, Lu S, Gu C, Guo JH (2011). A screening strategy of fungal biocontrol agents towards Verticillium wilt of cotton. Biol. Control, 56: 209-216.