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ANTIFUNGAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM EGYPTIAN HENBANE (*HYOSCYAMUS MUTICUS* L.)

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Abstract

A total of 44 strains of endophytic fungi have been identified from the medicinal plant *Hyoscyamus muticus* L. Ten of these strains were inoculated into aseptic seedlings of *H. muticus*. Of the inoculated strains, 9 were reisolated from the plants as endophytes. Using dual culture assay, we investigated whether eight of these strains, *Alternaria alternata*, *Aspergillus fumigatus*, *Drechslera hawaiiensis*, *Fusarium solani*, *Penicillium citrinum*, *Neoscytalidium dimidiatum*, *Thyrostromella myriana* and *Ulocladium chartarum* have antagonistic activities against two strains of plant pathogenic fungi viz., *Gibberella zeae* and *Thanatephorus cucumeris*, as well as 6 strains of non-pathogenic fungi viz., *Alternaria alternata*, *Cladosporium cladosporioides, Cladorrhinum foecundissimum, Curvularia clavata, Penicillium janthinellum* and *Ulocladium chartarum*. The endophytic fungi showed antagonistic activities against all the examined fungal strains. Extra- and intracellular fractions of *P. citrinum* and *N. dimidiatum* cultures, both of which showed high antagonistic activities, were extracted with ethyl acetate, *n*-butanol and water. The resulting fractions were examined for their antifungal activity by performing bioautography coupled with thin-layer chromatography (TLC-bioautography). Extracellular fluids secreted by the cultures of these organisms showed high antifungal activities against fungi, including the two plant pathogenic fungi; this result indicates that these endophytes secrete antifungal compounds extracellularly.

Introduction

In the past few decades, many studies have focused on endophytes—microorganisms that live inside plant tissues. These endophytes are present in all plants and are extremely abundant and often very diverse (Stone & Petrini, 1997; Schulthess & Faeth, 1998; Arnold *et al.*, 2000). They can survive in plants for all or part of their life without causing any apparent damage or diseases (Petrini, 1992). Endophytes can be transmitted vertically as well as horizontally: vertical transmission occurs through seeds and vegetative propagation of the host, and horizontal transmission occurs through spores, external to host tissues (Carroll, 1988). In the 1970's, endophytes were considered to be neutral: that is, they were believed to neither cause any harm nor benefit the plant. However, in the course of time, many studies revealed that endophytes play an important role in host protection against predators and pathogens (Azevedo *et al.*, 2000). In this case, the endophytic fungi live asymptomatically within the living tissue of the host plant and establish mutualistic symbiosis. Many are capable of synthesizing bioactive compounds that can be used by the plant for defense against pathogenic microorganisms (Schulz *et al.*, 2002; Strobel, 2003; Corrado & Rodrigues, 2004; Owen & Hundley, 2004; Giménez *et al.*, 2007).

Egyptian henbane (*Hyoscyamus muticus* L.), grown in the arid areas of Egypt, is a valuable medicinal plant for the production of tropane alkaloids, hyoscyamine and

scopolamine. In nature, many fungal species, including endophytic fungi, reside in *H. muticus* plants (El-Zayat *et al*., 2008). There is little information regarding the role of endophytic fungi in *H. muticus* plants. Recently, we found that *H. muticus* plants contain antifungal compounds other than alkaloids (Abdel-Motaal, unpublished data). Although these compounds are produced by *H. muticus* as secondary metabolites, they may contain compounds produced by the endophytic fungi that reside in the plant. As a first step toward verifying this hypothesis, we examined whether endophytic fungi isolated from *H. muticus* produce antifungal compounds *In vitro*.

Materials and Methods

Plant materials: Endophytic fungi were isolated from *H. muticus* plants growing (1) in a natural habitat in a desert in southern Egypt, (2) in a greenhouse in Japan (temperature, 10–37°C; humidity, 50–100%; and light, 10–14 h), or (3) in a growth chamber in Japan (temperature, 28°C; humidity, 55%; and photoperiod, 18 h light/6 h dark).

Isolation and identification of endophytic fungi: The roots, stems, and leaves of the *H. muticus* plants were separated. All the organs were surface sterilized in 70% (v/v) ethanol for 1 min and then in 5% (v/v) sodium hypochlorite solution for 5 min. They were subsequently washed twice with sterilized distilled water and blotted with sterilized filter papers (Rossman *et al*., 1998). The organs were longitudinally cut into 0.5–1 cm sections, and the resultant specimens were directly placed on a sterilized Petri dish containing corn meal agar. Four replicate plates were incubated at 28°C for 2–3 weeks. The fungi were identified on the basis of their morphological characteristics (Raper & Thom, 1949; Raper & Fennell, 1965; Ellis, 1971; Elis, 1976; Booth, 1977; Christensen *et al*., 1978; Pit, 1979; Pit, 1985; Moubasher, 1993).

rDNA sequence analysis: Nuclear DNA from isolates (*Penicillium citrinum* and *Neoscytalidium dimidiatum*) that exhibited a high inhibitory effect was extracted using the Dr. GenTLE kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The D1/D2 regions of the 26S rRNA gene and the internal transcribed spacer (ITS) region of the rRNA gene were directly sequenced from the PCR products using the following primers pairs: NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (3′-GGTCCGTGTTTCAAGACGG-5′), and ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATGATATGC-3′), respectively. The PCR products were sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were determined using an AB1 prism 3100 genetic analyzer (Applied Biosystems). The sequences of the ITS regions have been deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers AB490817 (*N. dimidiatum*) and AB490819 (*P. citrinum*). The sequence data were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) available from the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA). The phylogenetic analysis was carried out on the basis of the alignment of the ITS sequence. Multiple alignments were performed using the ClustalX program (Thompson *et al*., 1997), and the alignments were analyzed using the SEAVIEW software (Galtier *et al*., 1996). Evolutionary distances were calculated using Kimura's model (Kimura, 1980). Phylogenetic analysis was performed using the neighbor-joining method (Saitou & Nei, 1987), and bootstrap analysis was performed on

1000 replicates (Felsenstein, 1985). The phylogenetic tree was rooted with *Gaeumannomyces graminis* var. *avenae* AY428777 as an outgroup species.

Preparation of aseptic seedlings: *H. muticus* seeds collected in Egypt were surface sterilized in 70% ethanol for 1 min and then in 5% sodium hypochlorite solution for 5 min. The seeds were subsequently washed twice with sterilized distilled water and blotted with sterilized filter papers. Ten to twelve seeds were placed in a sterilized bottle containing 20 ml solid Murashige & Skoog (MS) salt mixture medium with antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA). The bottles containing the seedlings were incubated in a growth chamber at 28°C with an 18 h light/6 h dark cycle. Germinants were transferred to new bottles containing the same medium and incubated for complete sterilization. After 1 month, the seedlings were transferred to new bottles containing MS medium without an antifungal agent and incubated for 3 weeks. The 3-week incubation was repeated twice to exclude any remaining antifungal effect.

Inoculation and reisolation experiments of endophytic fungi: Endophytic fungi were incubated in potato dextrose broth (PDB; Difco Laboratories, Detroit, MI) for 5–10 days. The fungal culture was filtrated through three layers of gauze cloth to remove mycelia. Spore concentrations were adjusted with distilled water to obtain a concentration of 1×10^6 spores/ml. For root inoculation, the roots of aseptic seedlings were soaked in fungal spore suspensions (1×10^6 spores/ml) of endophytic fungi for 30 min. For leaf inoculation, the leaves and stems of aseptic seedlings were sprayed with a spore suspension using a sprayer. The inoculated seedlings were transplanted to new sterilized bottles containing sterilized potting soil (Yosaku; Japan Agricultural Cooperatives, Tokyo, Japan). Four replicates were carried out for each inoculation. The bottles were incubated in a growth chamber at temperatures varying irregularly from 12.5°C to 35°C for 1 week. The bottles containing plants were transferred to a growth chamber (temperature, 28°C) where the plants were allowed to grow for 3 weeks. The plants were recovered and sterilized as described above. Endophytic fungi were subsequently isolated from the plants, as described above.

Study of antagonistic activity of endophytes: The antagonistic activity of endophytic fungi against eight fungal strains was studied. Two plant pathogenic fungi, *Gibberella zeae* and *Thanatephorus cucumeris*, which cause head blight of wheat and sheath blight of rice, respectively, were isolated from diseased plants in Japan. Six strains, viz., *Alternaria alternata*, *Ulocladium chartarum*, *Cladosporium cladosporioides*, *Cladorrhinum foecundissimum*, *Curvularia clavata* and *Penicillium janthinellum*, were isolated from the surface of *H. muticus* plants. We used the dual culture method in order to investigate whether endophytic fungi have antagonistic activity against these pathogenic and non-pathogenic fungi. Mycelium discs (diameter, 5 mm) of 4-day-old fungi were placed in three corners of a potato dextrose agar (PDA) plate. Each of the eight endophytic fungi was inoculated at the center of each PDA plate. The plates were incubated at 25°C for 5 days. The percentage of inhibition was calculated from the following equation:

Inhibition (%) = [(growth diameter in the control sample – growth diameter in the sample with treated endophytes) × 100]/growth diameter in the control sample. The experiment was repeated twice in triplicates.

A dual culture was also performed to test the antagonistic activities of two endophytic fungi *P. citrinum* and *N. dimidiatum* against 6 other endophytic fungi viz., *A. alternata*, *Aspergillus fumigatus*, *Drechslera hawaiiensis*, *Fusarium solani*, *Thyrostromella myriana* and *Ulocladium chartarum*, which were isolated from *H. muticus* plants.

Extraction of antifungal compounds: Fourteen-day-old cultures of *P. citrinum* and *N. dimidiatum* on PDB were filtrated through three layers of gauze cloth and subsequently through a membrane filter (pore size, 0.8 μm). The fungal cells that were trapped on the gauze cloth were used as the intracellular fraction and the filtrates, as the extracellular fraction. The fungal cells were ground in liquid nitrogen. Both the ground fungal cells and filtrates were extracted thrice with ethyl acetate. The ethyl acetate fraction was transferred to a new conical flask. *n*-Butanol was added to and mixed well with the residue. The *n*-butanol fraction was subsequently transferred to a new conical flask. This extraction procedure was repeated thrice. The remaining part of the fungal cells was extracted thrice with water. The ethyl acetate and *n*-butanol fractions were evaporated using a rotary evaporator and subsequently freeze-dried, while the water fraction was frozen and freeze-dried.

Antifungal-activity assay: Ten-day-old cultures of pathogenic and non-pathogenic fungi on PDA media were harvested by flooding with 5 ml sterilized distilled water and dislodged using an L-shaped glass rod. The fungal suspension was filtrated through three layers of gauze cloth, and the filtrate was centrifuged at 1500 ×g for 10 min. The resulting spores were resuspended in PDB at a final concentration of 1×10^6 spores/ml. Both extra and intercellular fractions obtained from the endophytic fungi *P. citrinum* and *N. dimidiatum* were examined for their antifungal activities against pathogenic and non-pathogenic fungi by performing bioautography coupled with thin-layer chromatography (TLC-bioautography). Each fraction (ethyl acetate, *n*-butanol, and aqueous fractions) of *P. citrinum* and *N. dimidiatum* was dissolved in 70% methanol at a concentration of 100 mg/ml; 10 μl of each solution was applied to a TLC plate. The TLC plate spotted with the solution was dried and sprayed with a fungal spore suspension. The plates were incubated on a sheet of wet filter paper in a petri dish at 25°C for 3–7 days. The diameter of the inhibition zone was measured.

Results and Discussion

Isolation of endophytic fungi: A total of 44 fungal strains were isolated from healthy tissues of *H. muticus* plants grown under three different conditions: 30 from natural habitats, 9 from the green house, and 15 from the growth chamber (Table 1). To our knowledge, this is the first report describing the isolation of endophytic fungi residing in *H. muticus* plants. Of the isolated organisms, those belonging to the *Aspergillus* and *Penicillium* genera were predominant (11 and 5 species, respectively). Although these two genera are ubiquitous and usually epiphytic, they are known to grow endophytically as well (Schulthess & Faeth, 1998). Moreover, among the fungi isolated from *Melia azedarach*, the endophytic fungi belonging to *Aspergillus* and *Penicillium* were predominant (dos Santos *et al*., 2003). In the present study, *A. fumigatus* was found to be the most common species that was obtained from all plant tissues and different habitats.

Table 1. List of endophytic fungi isolated from root, stem and leaf of *Hyoscyamus muticus* plant growing in different habitats.

Fungal species	Growing place and plant organ		
	Natural habitat	Green house	Growth chamber
Acremonium strictum			leaf
Alternaria alternata	root, stem, leaf	stem	
Aspergillus candidus	root		
A. flavus	root, stem, leaf		
A. fumigatus	root, stem, leaf	leaf	root, stem
A. nidulans	leaf		
A. niger	root, stem, leaf		leaf
A. ochraeous	leaf		
A. oryzae	leaf		
A. sydowi	root, leaf		
A. terreus	root, stem, leaf		
A. ustus	leaf		
A. versicolor	stem, leaf		
Chaetomium globossum	stem, leaf		
Cladosporium cladosporioides	leaf		
C. herbarum		leaf	
C. oxysporum	leaf		
Curvularia clavata	root		
C. lunata			stem
Drechslera hawaiiensis	leaf		
Fusarium camptoceras			stem
F. aquaeductum		leaf	
F. solani		stem	
F. subglutinans		leaf	
F. tricinctum		root	leaf
Gliocladium roseum			root
Humicola grisea	root		
Mucor hiemalis	root, stem		
M. racemosus	root		
Myrothecium roridum	root		
Paecilomyces lilacinus			leaf
Penicillium brevicompactum.			root, stem
P. citrinum	root, stem		root, leaf
P. chrysogenoum	stem		stem
P. funiculosum	root, leaf		stem, leaf
P. waksmanii	stem		
Rhizopus stolonifier	root, stem, leaf		
Rhizoctonia solani		root	stem
Thyrostromella myriana	leaf		
Neoscytalidium dimidiatum	stem		
Trichurus spiralis		root	leaf
Ulocladium atrum	leaf		
U. chartarum	root, leaf		
Verticillium nubilum			leaf

Among the isolates, 7 species (*Alternaria alternata*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Penicillium citrinum* and *Rhizopus stolonifer*) were obtained from all plant tissues, while 27 species were obtained from different tissues in a tissue-specific manner: 6 species (*Aspergillus candidus*, *Curvularia clavata*, *Gliocladium roseum*, *Humicola grisea*, *Mucor racemosus*, and *Myrothecium roridum*) were found specifically in the roots; 6 (*Curvularia lunata*, *Fusarium camptoceras*, *Penicillium chrysogenum*, *P. waksmani* and *Scytalidium dimidiatum*), in the stem; and 15 (*Acremonium strictum*, *Aspergillus nidulans*, *A. ochraceous*, *A. oryzae*, *A. ustus*, *Cladosporium cladosporioides*, *C. herbarum*, *C. oxysporum*, *Drechslera hawaiiensis*, *Fusarium aquaeductum*, *F. subglutinans*, *Paecilomyces lilacinus*, *Thyrostromella myriana*, *Ulocladium atrum* and *Verticillium nubilum*), in the leaf. This tissue-specific distribution of endophytic fungi has also been reported in another plant (Cao *et al.*, 2002).

26S rDNA and internal transcribed spacer analysis: We identified two fungal isolates as *P. citrinum* and *N. dimidiatum* on the basis of their morphological characteristics; this was further confirmed by performing a BLAST search analysis of the sequences of their D1/D2 26S rDNA and ITS regions (data not shown). The sequences of the ITS regions have been deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers AB490817 (*N. dimidiatum*) and AB490819 (*P. citrinum*). Most of the species belonging to Botryosphaeriaceae are known as plant pathogens that cause branch wilt, canker, and die-back diseases in a wide variety of trees (Wilson, 1970). However, many fungal species belonging to this family have been described as endophytes (Crous *et al.*, 2006). *Neoscytalidium* is an endophytic fungi isolated from healthy sap wood and the bark of endemic trees (Pavlic & Wingfield, 2008). To the best of our knowledge this is the first report on the isolation of *N. dimidiatum* as an endophyte from herbaceous plants, including *H. muticus*. The phylogenetic tree indicating a close relationship between the isolate AB 490817 and *N. dimidiatum* are shown in Fig. 1.

Inoculation experiments and reisolation of endophytes: We selected 10 strains (*A. alternata*, *A. fumigatus*, *D. hawaiiensis*, *F. solani*, *P. citrinum*, *R. solani*, *T. myriana*, *N. dimidiatum*, *V. nubilum*, and *U. chartarum*) for the inoculation of aseptic seedlings of *H. muticus*. We reisolated nine strains, excluding *V. nubilum*, from different plant tissues of *H. muticus* with no disease symptoms; we thus confirmed that these nine strains are endophytic fungi that can reside in *H. muticus*.

The *V. nubilum* strain, originally isolated from the leaf of a healthy *H. muticus* plant, was not reisolated from the leaves or stems of plants whose leaves were inoculated with the strain. Interestingly, *H. muticus* plants whose roots were inoculated with the strain showed wilt symptoms and ultimately died; this observation suggests that the *V. nubilum* strain exhibited characteristics typical of a pathogenic fungus that causes wilt disease. It is known that an endophyte in one plant may act as a pathogen in another plant depending on the balance between pathogenicity and endophytism of the microorganism in the different hosts (Tan & Zou, 2001). Thus, *V. nubilum* residing in a particular tissue (leaf) of the *H. muticus* plant may act as a pathogen in the same plant when inoculated artificially into another tissue (root) of the plant, since the balance between the pathogenicity and endophytism of *V. nubilum* may differ from that in leaf tissue.

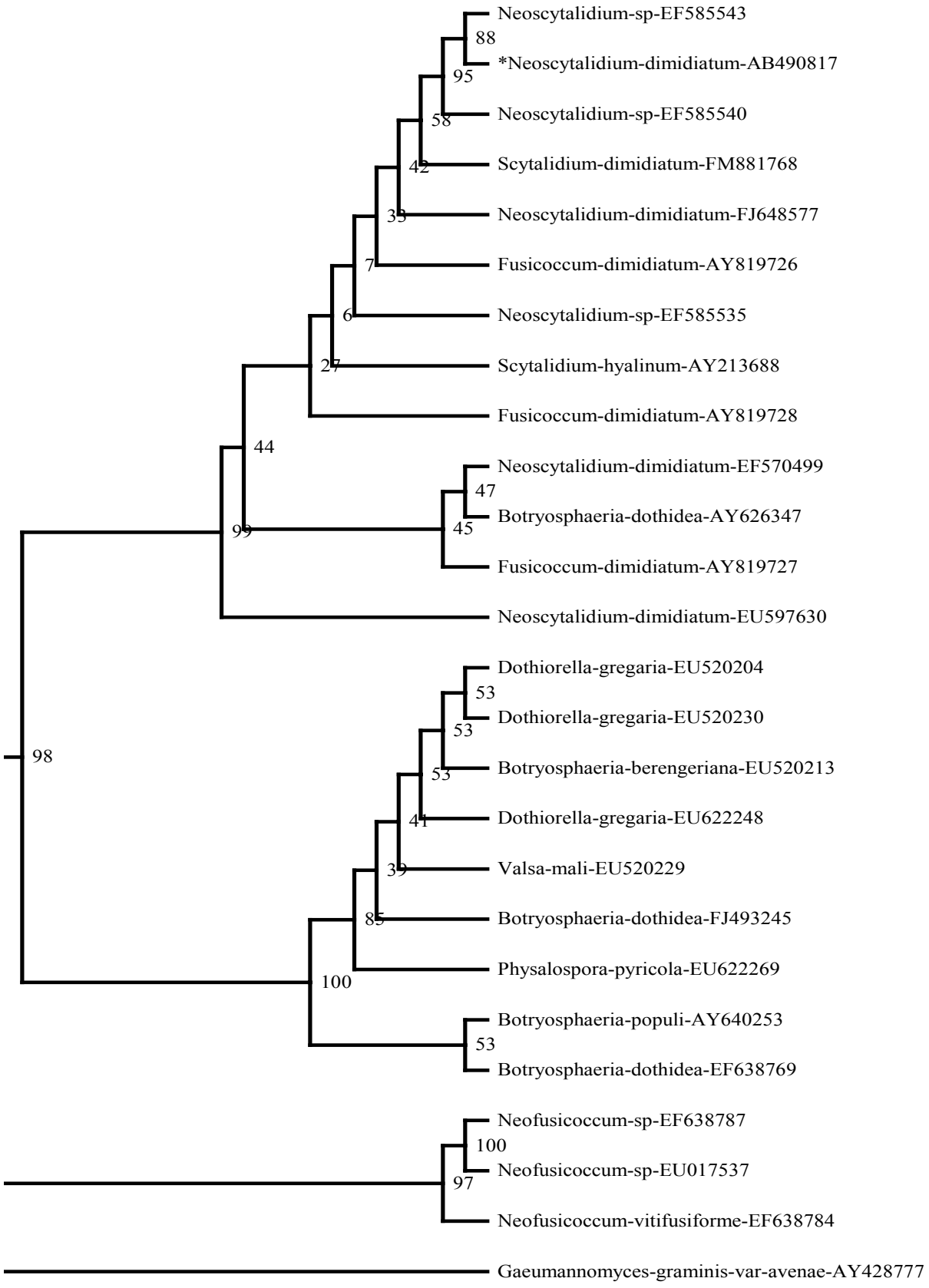

Fig. 1. Phylogenetic relationships between *Neoscytalidium dimidiatum* AB490817 and related taxa based on ITS regions. Clustering was performed with the neighbor-joining method using Kimura's model (Kimura, 1980) with the SEAVIEW software (Galtier *et al.*, 1996). The percentages at the nodes indicate the level of bootstrap pport of the branch point calculated from 1000 replicates. *Gaeumannomyces graminis* var. *avenae* AY428777 was used as an outgroup.

Antagonistic activity of endophytic fungi: Using the dual culture method, we examined eight endophytic fungi reisolated from *H. muticus* plants as endophytes for their antagonistic activities against 2 species of pathogenic fungi, *Gibberella zeae* and *Thanatephorus cucumeris* as well as 6 species of non-pathogenic fungi viz., *A. alternata*, *U. chartarum*, *C. cladosporioides, C. foecundissimum, C. clavata* and *P. janthinellum*. The endophytic fungi inhibited the growth of both pathogenic and non-pathogenic fungi (Fig. 2). *P. citrinum* followed by *N. dimidiatum* had higher antagonistic activity than the remaining six endophytic fungi. In particular, the antagonistic activity of *P. citrinum* was remarkable: 5 out of 8 fungi showed >50% reduction in growth. *N. dimidiatum* is known as a plant pathogen that causes branch wilt, canker, and die-back diseases in a wide variety of trees (Wilson, 1970). To the best of our knowledge, the antifungal activity of *N. dimidiatum* has not been reported, whereas the antifungal activities of the metabolites isolated from *P. citrinum* have been reported (Wakana *et al.*, 2006).

Our results suggest that the endophytic fungi residing in *H. muticus* plants produce antifungal compounds *In vitro*. The antagonistic activities of *P. citrinum* and *N. dimidiatum* against other endophytic fungi isolated from *H. muticus* plants were rather low compared to those against the 8 examined fungal strains (data not shown).

Antifungal activity of endophytic fungi: *P. citrinum* and *N. dimidiatum,* which showed higher antagonistic activity against a broad range of fungi, were examined for their antifungal activities by TLC-bioautography. Representative TLC-bioautography is shown in Fig. 3. The extracellular fractions of both the endophytic fungi were more effective than the intercellular fractions; which indicates that antifungal compounds produced by the endophytic fungi are secreted extracellularly (Table 2). Although there is no information regarding the secretion of antifungal compounds by endophytes such as *P. citrinum* and *N. dimidiatum* in *H. muticus* plants, antifungal compounds secreted extracellularly may contribute in part to the protection systems of the plant against pathogens. Further studies should be conducted in order to clarify whether the antifungal compounds of *P. citrinum* and *N. dimidiatum* are secreted in plants.

The intracellular ethyl acetate fraction of both the endophytes had high antifungal activity against a few fungal strains. This may be attributable to the antifungal compounds retained in cells, which are later secreted into the culture fluid. Moreover, endophytic fungi have been known to produce antifungal compounds extractable with ethyl acetate (Wicklow *et al.*, 2005).

It is interesting to note that *P. citrinum* and *N. dimidiatum* produced active antifungal compounds against the serious plant pathogenic fungi *T. cucumeris* and *G. zeae*, which are the causal agents of rice sheath blight and wheat head blight, respectively. Thus, these two strains of endophytic fungi may be potential biocontrol agents and sources of agricultural chemicals for rice sheath blight and wheat head blight, respectively. The remaining 34 strains of endophytic fungi isolated from *H. muticus* in the present study should be examined for detecting their antifungal activities against plant pathogenic fungi.

Acknowledgments

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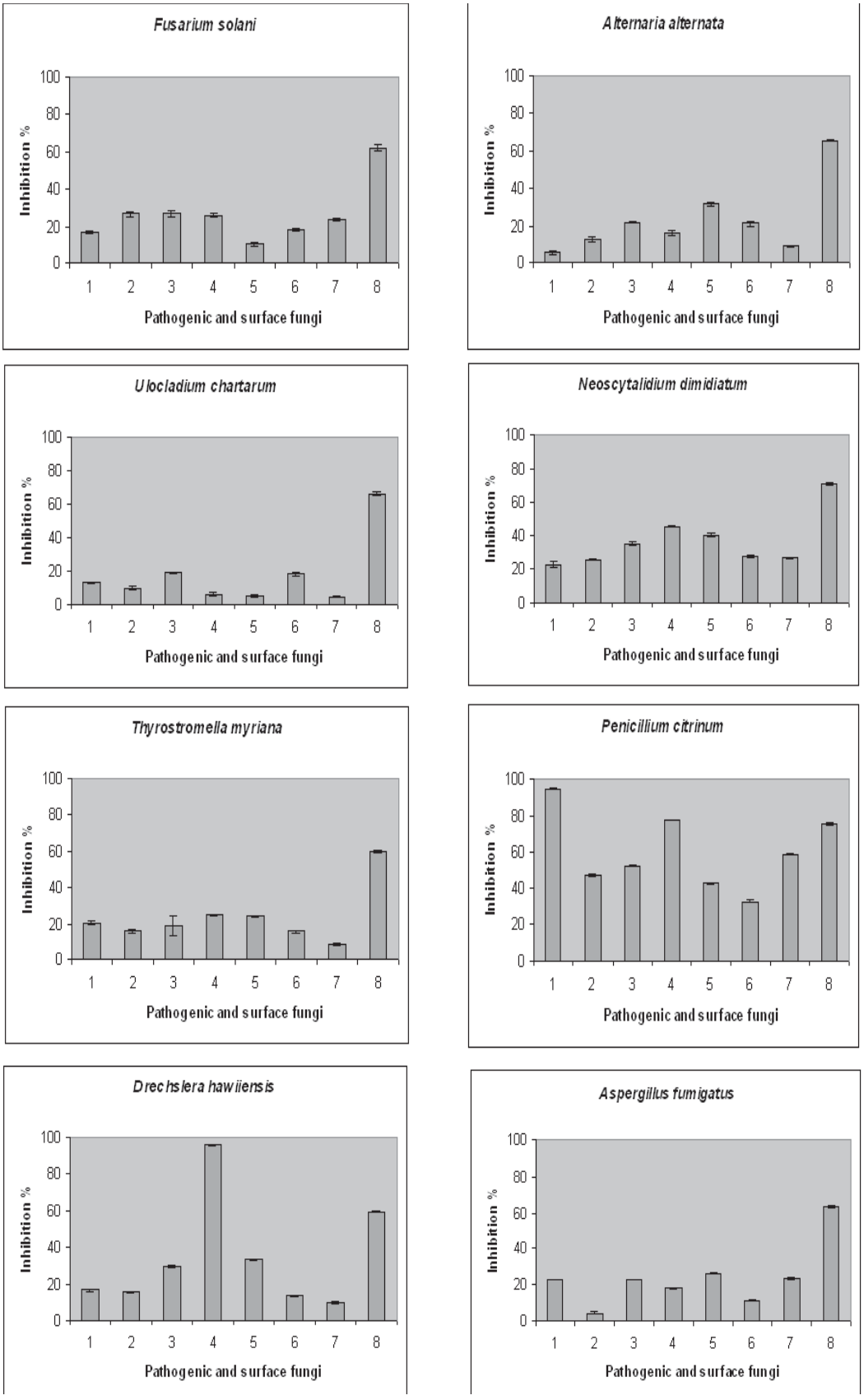

Fig. 2. Antagonistic activity of eight endophytic fungi reisolated from *H. muticus*. The eight endophytic fungi are shown in the upper part of each panel. 1, *Thanatephorus cucumeris*; 2, *Gibberella zeae*; 3, *Alternaria alternata*; 4, *Ulocladium chartarum*; 5, *Cladosporium cladosporioides*; 6, *Cladorrhinum foecundissimum*; 7, *Curvularia clavata*; and 8, *Penicillium janthinellum*. Data are presented as the mean ± S.E.M. (n = 6).

Table 2. Antifungal activity of secondary metabolites from *Penicillium citrinum* and *Neoscytalidium dimidiatu* detected by TLC-bioautography [a].

Fungal species examined	*Pencillium citrinum*						*Neoscytalidium dimidiatum*					
	Extracellular			Intracellular			Extracellular			Intracellular		
	E [b]	B	A	E	B	A	E	B	A	E	B	A
Alternaria alternata	23	25	–	15	25	–	21	5	–	12	6	–
Cladorrhinum focundissimum	19	25	–	12	–	–	16	20	–	23	–	–
Cladosporium cladosporioides	15	27	–	13	10	–	19	15	–	11	10	–
Curvularia clavata	14	27	–	–	–	–	12	12	–	15	–	–
Gibberella zeae	13	2	–	–	–	–	15	–	–	18	–	–
Penicillium janthinellum	15	28	–	9	–	–	9	23	–	12	–	–
Thanatephorus cucumeris	25	23	–	18	12	–	9	7	–	12	7	–
Ulocladium chartarum	12	18	–	14	18	–	12	13	–	–	–	–

[a]Values are diameter of inhibition zone (mm). No inhibition is denoted as "–". [b] E, ethyl acetate fraction; B, *n*-butanol fraction; A, aqueous fraction.

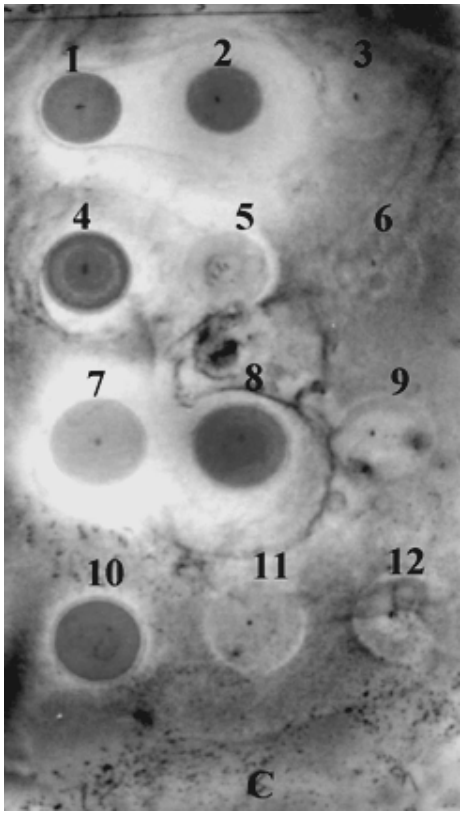

Fig. 3. TLC-bioautography of extracts of extra- and intracellular fractions of *Penicillium citrinum* and *Neoscytalidium dimidiatum* cultures. An aliquot (1 mg in 10 □l of 70% methanol) of extracellular ethylacetate (1 and 7), extracellular *n*-butanol (2 and 8), extracellular aqueous (3 and 9), intracellular ethylacetate (4 and 10), intracellular *n*-butanol (5 and 11), and intracellular aqueous (6 and 12) fractions of *P. citrinum* (1–6) and *N. dimidiatum* (7–12) cultures were applied to a TLC plate. The spotted TLC plates were dried and sprayed with *Cladosporium cladosporioides* and incubated at 25°C for 3 days. "C" denotes the negative-control spot (70% methanol).

References

Arnold, A.E, Z. Maynard, G.S. Gilbert, P.D. Coley and T.A. Kursar. 2000. Are tropical fungal endophytes hyperdiverse? *Ecol. Lett.*, 3: 267-274.

Azevedo, J.L., J.W. Maccheroni, J.O. Pereira and W.L. Araújo. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electr. J. Biotechnol.*, 3: 40-65.

Booth, C. 1977. *Fusarium*. Laboratory guide to the identification of major species. Commonwealth Mycol Inst, Kew, Surrey.

Cao L.X., J.L. You and S.N. Zhou. 2002. Endophytic fungi from *Musa acuminate* leaves and roots in South China. *World J. Microbiol. Biotechnol.*, 18: 169-171.

Carroll, G. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology*, 69: 2-9.

Christensen, M. and K.B. Raber. 1978. Synoptic key to *Aspergillus nidulans* group species and related *Emericella* species. *Trans. Br. Mycol. Soc.*, 71: 177-191.

Corrado, M. and K.F. Rodrigues. 2004. Antimicrobial evaluation of fungal extracts produced by endophytic strains of *Phomopsis* sp. *J. Basic Microbiol.*, 44: 157-160.

Crous, P.W., B. Slippers, M.J. Wingfield, J. Rheeder, W.F.O. Marasas, A.J.L. Phillips, A. Alves, T. Burgess, P. Barber and J.Z. Groenewald. 2006. Phylogenetic lineages in the Botryosphaeriaceae. *Studies Mycol.*, 55: 239-57.

dos Santos, R.M.G., E. Rodrigues-Fo, W.C. Rocha and M.F.S. Teixeira. 2003. Endophytic fungi from *Melia azedarach. World J. Microbiol. Biotechnol.*, 19: 767-770.

Ellis, M.B. 1971. *Dematiaceous Hyphomycetes*. Commonwealth Mycol Inst, Kew, Surrey, p. 608.

Ellis, M.B. 1976. *More Dematiaceous Hyphomycetes*. Commonwealth Mycol Inst, Kew, Surrey, p. 507.

El-Zayat, S.A., M.S. Nassar, F.T. El-Hissy, F.F. Abdel-Motaal and S. Ito. 2008. Mycoflora associated with *Hyoscyamus muticus* growing under an extremely arid desert environment (Aswan region, Egypt). *J. Basic Microbiol*., 48: 82-92.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39: 783-791.

Galtier, N., M. M. Guy and C. Gautier. 1996. SEAVIEW and PHYLO WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.*, 12: 543-548.

Giménez, C., R. Cabrera, M. Reina and González-Coloma. 2007. Fungal endophytes and their role in Plant Protection. *Curr. Org. Chem.*, 1: 707-720.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, 16: 111-120.

Moubasher, A.H. 1993. *Soil fungi in Qatar and other Arab countries*. The scientific and applied research center, University of Qatar, Doha, p. 566.

Owen, N.L. and N. Hundley. 2004. Endophytes-the chemical synthesizers inside plants. *Sci. Progress*, 87: 79-99.

Pavlic, D. and J.M. Wingfield. 2008. Seven new species of the Botryosphaeriaceae from baobab and other native trees in Western Australia. *Mycologia*, 100: 851-866.

Petrini, O, T.N. Sieber, L. Toti and O. Viret. 1992. Ecology, metabolite production and substrate utilization in endophytic fungi. *Nat. Toxins*, 1: 185-196.

Pitt, J.I. 1979. The genus *Penicillium* and its telemorphic stato *Eupenicillium* and *Talaromyces*. Academic Press, London.

Pitt J.I. 1985. A laboratory guide to common *Pencillium* species. Commonwealth scientific and Industrial Research Organization, Division of food research, North Ryde.

Raper, K.B. and D.I. Fennell. 1965. The genus *Aspergillus*. Williams and Wilkins, Baltimore, p. 686.

Raper, K.B. and C.A. Thom. 1949. A manual of the *Penicillium,* Williams and Wilkins, Baltimore, p. 875.

Rossman, A.Y., R.E. Tulloss, T.E. O'Dell and R.G. Thorn. 1998. *Protocols for an all taxa biodiversity inventory of fungi in Costa Rican conservation area*. Parkway publishers, Boone, p. 218.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.

Schulthess, F.M. and S.H. Faeth. 1998. Distribution, abundances, and associations of the endophytic fungal community of Arizona fescue (*Festuca arizonica*). *Mycologia*, 90: 569-578.

Schulz, B., C. Boyle, S. Draeger, A.K. Römmert and K.Krohn. 2002. Endophytic fungi. A source of novel biologically active secondary metabolites. *Mycol. Res.*, 106: 996-1004.

Stone, J.K. and O. Petrini. 1997. Endophytes of forest trees: a model for fungus-plant interactions. In: *The Mycota*. (Eds.): G.C. Carroll & P. Tudzynski. V. Part B. Plant relationships, Springer-Verlag, Berlin, pp. 129-142.

Strobel, G.A. 2001. Endophytes as sources of bioactive products. *Microbes Infect.*, 5: 535-544.

Tan, R.X. and W.X. Zou. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.*, 18: 448-459.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25: 4876-4882.

Wakana, D., T. Hosoe, T. Itabashi, K. Okada, G.M.C. Takaki, T. YAguchi, K. Fukushima and K. Kawai. 2006. New citrinin derivatives isolated from Penicillium citrinum. *J. Nat. Med*., 60: 279-284.

Wicklow, D.T., S. Roth, S.T. Deyrup and J.B. Gloer. 2005. A protective endophyte of maize: Acremonium zeae antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. *Mycol. Res.*, 109: 610-618.

Wilson, E.E. 1970. The branch wilt of Persian walnut trees and its cause. *Hilgardia*, 17: 413-430.

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