The Journal of Microbiology (2012) Vol. 50, No. 4, pp. 581–585
DOI 10.1007/s12275-012-2083-8

Enhancement of Anti-candidal Activity of Endophytic Fungus *Phomopsis* sp. ED2, Isolated from *Orthosiphon stamineus* Benth, by Incorporation of Host Plant Extract in Culture Medium

Tong Woei Yenn[1]*, Chong Chai Lee[1], Darah Ibrahim[1], and Latiffah Zakaria[2]

[1]Industrial Biotechnology Research Laboratory, [2]Plant Pathology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

(Received February 14, 2012 / Accepted April 23, 2012)

This study examined the effect of host extract in the culture medium on anti-candidal activity of *Phomopsis* sp. ED2, previously isolated from the medicinal herb *Orthosiphon stamineus* Benth. Interestingly, upon addition of aqueous host extract to the culture medium, the ethyl acetate extract prepared from fermentative broth exhibited moderate anti-candidal activity in a disc diffusion assay. The minimal inhibitory concentration of this extract was 62.5 μg/ml and it only exhibited fungistatic activity against *C. albicans*. In the time-kill study, a 50% growth reduction of *C. albicans* was observed at 31.4 h for extract from the culture incorporating host extract. In the bioautography assay, only one single spot (R_f 0.59) developed from the extract exhibited anti-candidal activity. A spot with the a similar R_f was not detected for the crude extract from YES broth without host extract. This indicated that the terpenoid anti-candidal compound was only produced when the host extract was introduced into the medium. The study concluded that the incorporation of aqueous extract of the host plant into the culture medium significantly enhanced the anti-candidal activity of *Phomopsis* sp. ED2.

Keywords: endophytes, *Phomopsis* sp., *Orthosiphon stamineus* Benth, *Candida albicans*, host plant extract

Introduction

There is a need for anti-candidal drugs due to the increasing opportunistic fungal infections in immunocompromised patients. *Candida albicans* is the fourth most common cause of systemic mycoses. In the past two decades, the frequency of candidiasis has increased ten-fold (Fostel and Lartey, 2000; Gupte *et al.*, 2002; Kim and Sudbery, 2011). There is a need for new anti-candidal compounds from natural sources, which can then serve as templates for further chemical modifications.

To date, the search for bioactive compounds has focused mainly on soil microorganisms; however, due to the rapid decrease in discovering microorganisms that produced new metabolites, an exploration for new biotopes is necessary (Schulz *et al.*, 2002; Guo *et al.*, 2011). Endophytes can be defined as the microorganisms that colonize healthy plant tissue and cause no disease symptoms for their host. They are ubiquitous and have been found residing asymptomatically in almost all living plant tissues (Strobel, 2003; Yu *et al.*, 2010). Hence, they are expected to have high potential as sources of new natural bioactive compounds and plant metabolites (Tan and Zou, 2001; Mourad, 2010).

Phomopsis is a genus that includes over 1000 species classified on the basis of their host plant, and is frequently isolated as an endophyte (Farr *et al.*, 2002). It is also well known for the production of bioactive compounds that exhibit antimicrotubule (Sreekanth *et al.*, 2011), antimalarial (Tansuwan *et al.*, 2007), antitubercular (Castillo *et al.*, 2002), antifungal (Weber *et al.*, 2007; Ding *et al.*, 2008), herbicidal (Qiu *et al.*, 2006), algicidal (Gond *et al.*, 2007), anti-inflammatory (Nithya and Muthumary, 2011), antimicrobial, and plant growth regulatory activities (Horn *et al.*, 1995; Carrado and Rodrigues, 2004; Rukachaisirikul *et al.*, 2007; Huang *et al.*, 2008). These bioactive compounds include phomopsin, phomopsidin, phomoxanthones, phomoenamide, phomonitroester, diacetylphomoxanthone, phomodiol, diaryl ethers, phomosines, phomalactone, phomopsichalasin, and cytochalasin (Rukachaisirikul *et al.*, 2007; Huang *et al.*, 2008). Numerous reports are available on the antimicrobial activity of *Phomopsis* sp. (Horn *et al.*, 1995; Carrado and Rodrigues, 2004; Rukachaisirikul *et al.*, 2007; Huang *et al.*, 2008). In previous studies, antimicrobial compounds such as phomoxanthone A and phomopsichalasin, have been isolated from *Phomopsis* sp. (Rukachaisirikul *et al.*, 2007).

The production of antibiotics by filamentous fungi can be enhanced by gene amplication, genetic modification, mixed culture fermentation, immobilization of the cells, optimization of fermentation conditions, or enzymes induction (Oyama and Kubota, 1993; Ho *et al.*, 2003). Endophytes have a symbiotic relationship with their hosts. Increasing evidence suggests that this symbiotic relationship enables the host plants to produce nutrients for the endophytes whilst the endophytes enhance the survivability of the host by producing bioactive metabolites (Tan and Zou, 2001; Tong *et al.*, 2011). Although the symbiotic relationship is clear, little is known about the role of host extract on the biological activity of endophytes. Hence, in this study, we examined the

*For correspondence. E-mail: woeiyenn@yahoo.com; Tel.: +6016-4822046; Fax: +604-6565125

effect of host plant extract in the culture medium on the anti-candidal activity of *Phomopsis* sp. ED2, an endophytic fungus isolated from the medicinal herb *Orthosiphon stamineus* Benth.

Materials and Methods

Endophytic fungus and storage

The endophytic fungus *Phomopsis* sp. ED2 previously isolated from *O. stamineus* Benth was deposited at the Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultivated on Potato Dextrose Agar (PDA; AES) supplemented with powdered host plant materials (5 g/L) and stored at 4°C prior to use. The isolate was subcultured on fresh medium every four weeks to ensure purity and viability.

Culture media

Yeast extract sucrose (YES) broth (yeast extract 20 g/L, sucrose 40 g/L, magnesium sulfate 0.5 g/L) was used to cultivate *Phomopsis* sp. ED2 in shake-flasks, with and without the aqueous extract of *O. stamineus*. Healthy host plant materials were collected from Balik Pulau, Penang, Malaysia from an area free of fungicides. The plant extract was prepared by boiling 10 g of dried plant material in 500 ml distilled water for 30 min. The extract was filtered and mixed with freshly prepared culture medium and autoclaved at 121°C for 15 min.

Fermentation and extraction

The inoculum was prepared by introducing two mycelial agar plugs into 250 ml Erlenmeyer flasks containing 100 ml of YES medium. Both agar plugs were 1 cm in diameter, 4 mm thick and excised from the periphery of a 7-days-old fungal culture. The cultures were grown at 30°C in a shaker at 120 rpm. After 20 days of incubation, the fermentation broth and fungal biomass were separated by centrifugation at 5311×g (Sigma, Model 4K15, Germany). The fungal biomass was soaked in methanol (1:50, w/v) overnight. The supernatant was then extracted thrice with an equal volume of ethyl acetate (1:1, v/v). The upper organic phase was concentrated to a crude extract paste using a rotary evaporator under reduced pressure. Due to the antimicrobial activities exhibited by *O. stamineus*, a control was prepared by using sterile medium following the same procedure used for fungal cultures.

Test isolate

The *C. albicans* culture which was previously isolated from a clinical sample taken in Hospital Seberang Jaya, Penang, Malaysia was provided by the Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The culture was grown on Sabouraud Dextrose Agar (SDA) plates (Merck) at 37°C. The inoculum suspension was prepared by picking five single colonies from a 24-h-old culture and putting them into 5 ml of sterile physiological saline. The turbidity of the inoculum suspension was adjusted with sterile saline to match the turbidity of a 0.5 Mc-Farland standard.

Anti-candidal susceptibility testing

The assay was performed according to the method of Espinel-Ingroff *et al.* (1992). Crude extracts were dissolved in 5% dimethyl sulfoxide (DMSO). Using a sterile cotton swab, tested inoculum of approx 1×10^6 CFU/ml was streaked onto the surface of a Mueller-Hinton agar plate (Hi-media) containing 2% dextrose and 0.5 µg/ml methylene blue. Sterile Whatman antibiotic discs impregnated with 20 µl of fungal extracts at a concentration of 20 mg/ml were then placed on the surface of the inoculated medium. A 5% DMSO solution was applied as a negative control to measure the solvent effects, and 30 µg/ml amphotericin B was used as a positive control. The plates were incubated at 37°C for 24 h and the diameter of the clear zone was measured. The results were expressed as mean value±standard error of the inhibition zone obtained with three replicates for each experiment.

MIC and MFC determination

The minimal inhibitory concentration (MIC) of the fungal extract was determined by a broth microdilution assay in RPMI 1640 medium (Sigma) containing 0.2% dextrose buffered with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS) to a pH of 7.0 at 25°C. The broth microdilution assay was conducted in a sterile, 96-wells, U-shaped, microtiter plate according to Jorgensen and Turnidge (2007). A two-fold dilution of the fungal extract was prepared with sterile medium and 100 µl of the extract was dispensed into each well on the microtiter plate. On the same day, 100 µl of yeast inoculum at approx 1×10^3 CFU/ml yeast cells was added to each well, for a final vol of 200 µl. Amphotericin B was used as the reference drug, and a control with 5% DMSO and yeast inoculum was included. After a 24 h-incubation at 37°C in a rotary shaker, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet salt (INT) (Sigma) dissolved in 99.5% ethanol was added to each well as a growth indicator. The color of INT changed from yellow to purple where microbial growth occurred. The MIC was recorded as the lowest concentration of extract that inhibited the growth of yeast. The minimal fungicidal concentration (MFC) of the extract was also determined. After the MIC was read at 24 h, the viable cells were enumerated on SDA plates by a standard plate count. Inoculated plates were then incubated at 37°C for 24 h. The MFC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control.

Time-kill study

One-way Analysis of Variance (ANOVA) was used to determine the statistical difference between the effects of different fungal extracts, on the growth of *C. albicans*. A dilution of the inoculum was prepared by transferring 5 ml of yeast inoculum into 45 ml of RPMI 1640 medium (Sigma) containing 0.2% dextrose and buffered with 0.165 M MOPS to a pH of 7.0 at 25°C, with or without fungal extract. This dilution yielded a starting inoculum cell density of approx 1×10^5 CFU/ml. The cultures were then incubated at 37°C in a rotary shaker at 150 rpm for 48 h. At predetermined time points (0–48 h

with 4 h of intervals), a 1 ml sample was removed from the flask and diluted 10-fold with sterile saline. The aliquot was spread onto an SDA plate for viability determinations. Inoculated plates were then incubated at 37°C for 24 h. A flask with 5% DMSO served as a control. A time-kill curve (log CFU/ml vs. time) was drawn for each extract, including sterile broth, and linear regression analysis was performed for each set of data. Experiments were performed in triplicate on separate occasions.

Bioautography assay

The inhibitory activity of fungal extracts on *C. albicans* was investigated by thin layer chromatography (TLC) using the bioautographic agar overlay method (Valgas *et al*., 2007). Five microliters of different extract preparations were applied to a TLC aluminium sheet plate (Merck). The plates were then developed with a chloroform:methanol (9:1) solvent system and thoroughly dried overnight to achieve complete removal of solvents. The developed TLC plates were observed under visible light and UV light at 254 nm and 366 nm, respectively. The TLC plate was then placed on the surface of Mueller-Hinton agar plate containing 2% dextrose and 0.5 µg/ml methylene blue. Then, the same molten agar medium inoculated with *C. albicans* was poured onto the TLC plate. The plates were then incubated at 37°C for 24 h. The microbial growth inhibition appeared as clear zones around the active spot. To perform phytochemical analysis of the active spot, the developed TLC plate was kept in a closed iodine chamber to visualize the separated compound. In addition, another newly developed TLC plate was sprayed with 1% vanillin sulfuric acid reagent and heated gently as the comfirmation test for terpenoids.

Preparative TLC and anti-candidal activity of partially purified compound

To obtain the partially purified compound, a preparative TLC plate was activated at 100°C for 15 min. The ethyl acetate extract from YES culture with host extract was applied on the plate and developed with a chloroform:methanol (9:1, v/v) solvent system. After separation, the band with R_f 0.59 was scrapped and mixed with methanol. After centrifugation at 5,000 rpm for 15 min, the supernatant was collected and dried. The dried paste was used for the anti-candidal assay mentioned in section 2.5 and 2.6.

Table 1. MIC and MFC values for the effect of the ethyl acetate extract from YES with host extract and with Amphotericin B on *C. albicans*

Microorganism	MIC (µg/ml)		MFC (µg/ml)	
	EP	Amphotericin B	EP	Amphotericin B
C. albicans	62.5	0.98	1000	1.95

*EP- Ethyl acetate extract from YES with host extract

Results and Discussions

On the disc diffusion assay, none of the crude extracts prepared from the fungal biomass exhibited anti-candidal activity, for the two culture media. For the fermentative broth, the ethyl acetate extract of the culture supplemented with host plant extract showed a clear inhibition zone with a diameter of 17.7±0.6 mm surrounding the paper disc. In contrast, no clear zone was observed for the ethyl acetate extract from the culture without host extract. Interestingly, upon addition of aqueous host extract to the culture medium, the ethyl acetate extract prepared from fermentative broth exhibited moderate anti-candidal activity. This may be due to the need for certain host plant compounds, which may serve as precursors, for the biosynthesis of secondary metabolites in endophytic fungi (Tong *et al*., 2011). These findings also suggest mutualistic interaction between the endophytic fungus and its host, where the fungus produces anti-candidal metabolites using plant nutrients.

The susceptibility test results for ethyl acetate extract, prepared from YES culture with host extract, are presented in Table 1. The MIC value of the ethyl acetate extract which is significantly higher than the MFC value, indicates that a

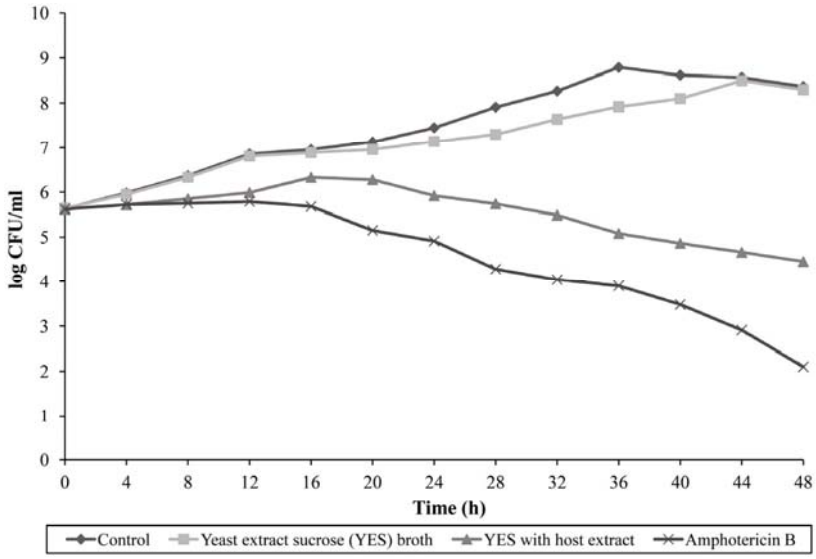

Fig. 1. Time-kill curves of amphoterin B and various extracts against *C. albicans*.

Table 2. Time to achieve 25, 50, 90, and 99.9% growth reductions for *C. albicans* from starting inoculum

Growth reduction (%)	Median time (h)			
	Control	YES	YES with host extract	Amphotericin B
25%	NR	NR	25.6	11.5
50%	NR	NR	31.4	13.8
90%	NR	NR	NR	23.0
99.9%	NR	NR	NR	23.2

* NR, not reached

higher concentration of extract was needed to kill the yeast cells, than to inhibit the growth. Amphotericin B exhibited fungicidal activity against *C. albicans* with a low MFC of 1.95 μg/ml. The anti-candidal activity of the ethyl acetate extract of *Phomopsis* sp. ED2 cultivated in YES broth with host extract was significantly lower ($p<0.05$) than that of amphotericin B. The extract exhibited only fungistatic activity and it did not kill the *C. albicans* cells as the MFC value was 16 times higher than its MIC value. No inhibitory activity can be detected in the extract from the culture without the host extract. Hence, this provides concrete evidence that the presence of host extract in the culture medium significantly enhances the anti-candidal activity by *Phomopsis* sp. ED2.

In the time-kill study, the killing patterns of amphotericin B and the fungal extracts against *C. albicans* were similar to the MIC and MFC results. Figure 1 shows that the time-kill curve of the control (5% DMSO) and ethyl acetate extracts from YES broth culture showed normal growth phases, and high colony counts were obtained at the end of the incubation. Based on Table 2, only amphotericin B exhibited fungicidal activity at 23.2 h; 99.9% of the yeast cells were killed and a low colony count was obtained at 48 h compared to the control. In contrast, none of the fungal extact exhibited fungicidal activity against *C. albicans*, mirroring the susceptibility test results. No notable difference can be observed for the time-kill curve of control and the extract from culture without host extract. However, it is noteworthy that a significant difference was observed between fungal extracts, cultivated in YES broth with and without host extract ($p<0.05$). With the addition of host extract to the culture medium, a 50% growth reduction was observed at 31.4 h, whereas this was not achieved by the medium alone without plant extract. The anti-candidal effect of the culture with host extract is significant as the colony count obtained throughout the whole incubation period was significantly lower than for the culture without host extract, indicating the anti-candidal effect can only be observed when the host plant extract was added to the culture medium of *Phomopsis* sp. ED2.

Liu *et al.* (2004, 2010) reported the isolation of two new bioactive metabolites, ethyl 2,-dihydroxy-5,6-dimethylbenzoate and phomopsilactone from an endophytic fungus *Phomopsis cassiae* from *Cassia spectabilis* and the compounds displayed strong antifungal activity. However, in the present study, the anti-candidal activity was observed only when the host extract was incorporated into the culture medium. Therefore, the presence of host compounds in the medium plays an important role in the biological activity of the endophytic fungus. The results showed that the anti-candidal activity was solely due to the presence of the anti-candidal compounds resulting from a biotransformation process by *Phomopsis* sp. ED2.

The TLC results indicated that the crude extract from YES broth culture containing the host extract showed the highest number of spots, including three spots in visible light, two spots under 254 nm UV light and three spots under 366 nm UV light. This indicates that the presence of host extract in the culture medium enhances the production of metabolites of *Phomopsis* sp. ED2. In the bioautography assay, the extract prepared from YES culture containing host extract produced one spot with an R_f of 0.59 and exhibited inhibitory activity on *C. albicans*. The activity was mild compared to amphotericin B. A spot with a similar R_f was not observed with the crude extract from YES broth without host extract, nor with the extract of sterile medium. This indicates that the anti-candidal compound can only be produced when the host extract was introduced into the medium.

For phytochemical analysis of the spot with anti-candidal activity, the TLC plate was subjected to UV light and the spot showed bluish color under 366 nm UV light and when it was subjected to iodine vapour, it showed a brown spot at R_f 0.59. It was further confirmed with spray reagent vanillin-sulfuric acid, which gave a blue spot at R_f 0.59. The results showed that the compound was terpenoid in nature.

The partially purified terpenoid compound showed an inhibition zone of 14.3 mm against *C. albicans* seeded agar on the disc diffusion assay. This diameter was smaller than that for the crude extract. This was possibly due to the synergistic interactive effect of the compounds present in the crude extract. In the broth microdilution assay, the partially purified compound showed a MIC value of 125 μg/ml, mirroring the result from the disc diffusion assay, where the crude extract showed greater anti-candial activity. However, the MFC value of the partially purified compound on *C. albicans* is 1,000 μg/ml, showing no significant difference in the candida-killing capability with crude extract.

Conclusion

Our findings show that *Phomopsis* sp. ED2, an endophytic fungus of *O. stamineus*, has antimicrobial, especially anti-candidal properties. Even though the role of the endophytic fungus is still not very clear, it likely brings benefits to the host plant. This study also demonstrated that with the addition of host extract, the anti-candidal activity of the endophytic fungus *Phomopsis* sp. ED2 can be enhanced. This is an important milestone for further studies of endophytic fungi, as it suggests a close interaction of the endophytic microorganisms with their hosts.

Acknowledgements

The authors are thankful to National Science Fellowship and USM post-graduate grant scheme for the research grant and financial support in this study.

References

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

Castillo, U.F., Strobel, G.A., Ford, E.J., Hess, W.M., Porter, H., Jensen, J.B., Albert, H., Robinson, R., Condron, M.A.M., Teplow, D.B., and *et al.* 2002. Munumbicins, wide spectrum antibiotics produced by *Streptomyces* NRRL 30562, endophytic on *Kennedia nigriscans*. *Microbiology* **148**, 2675–2685.

Ding, G., Liu, S., Guo, L., Zhou, Y., and Che, Y. 2008. Antifungal metabolites from the plant endophytic fungus *Pestalotiopsis foedan*. *J. Nat. Prod.* **71**, 615–618.

Espinel-Ingroff, A., Kish, J.C.W., Kerkering, T.M., Fromtling, R.A., Bartizal, K., Galgiani, J.N., Villareal, K., Pfaller, M.A., Gerarden, T., and Rinaldi, M.G. 1992. Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. *J. Clin. Microbiol.* **30**, 3138–3145.

Farr, D.F., Castlebury, L.A., and Rossman, A.Y. 2002. Morphological and molecular characterization of *Phomopsis vaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in the eastern United States. *Mycologia* **94**, 494–504.

Fostel, J.M. and Lartey, P.A. 2000. Emerging novel antifungal agents. *Ther. Focus* **5**, 25–32.

Gond, S.K., Verma, V.C., Kumar, A., Kumar, V., and Kharwar, R.N. 2007. Study of endophytic fungal community from different parts of *Aegle marmelos* Correae (Rutaceae) from Varanasi (India). *World J. Microb. Biot.* **23**, 1371–1375.

Guo, Z., Hua, R., Bai, Y., Wu, X., Cao, H., Li, X., Wu, Y., and Tang, J. 2011. Screening and evaluation of antiphylopathogenic activity of endophytic fungi from live foliages of *Ginkgo biloba* L. *Afr. J. Microbiol. Res.* **5**, 1686–1690.

Gupte, M., Kulkarni, P., and Ganguli, B.M. 2002. Antifungal antibiotics. *Appl. Microbiol. Biotechnol.* **58**, 46–57.

Ho, W.H., To, P.C., and Hyde, K.D. 2003. Induction of antibiotic production of freshwater fungi using mix-culture fermentation. *Fungal Divers.* **12**, 45–51.

Horn, W.S., Simmonds, M.S.J., Schwartz, R.E., and Blaney, W.M. 1995. Phomopsichalasin, a novel antimicrobial agent from an endophytic *Phomopsis* sp. *Tetrahedron* **51**, 3969–3978.

Huang, Z., Cai, X., Shao, C., She, Z., Xia, X., Chen, Y., Yang, J., Zhou, S., and Lin, Y. 2008. Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. *Phytochem.* **69**, 1604–1608.

Jorgensen, J.H. and Turnidge, J.D. 2007. Susceptibility test methods: dilution and disk diffusion methods, pp. 1152–1172. *In* Murray, P.R., Baron, E.J., Jorgensen, J.H., Landry, M.L., and Pfaller, M.A. (eds.), Manual of Clinical Microbiology. ASM Press, Washington, D.C., USA.

Kim, J. and Sudbery, P. 2011. *Candida albicans*, a major human fungal pathogen. *J. Microbiol.* **49**, 171–177.

Liu, C.H., Liu, T.T., Yuan, F.F., and Gu, Y.C. 2010. Isolating endophytic fungi from evergreen plants and determining their antifungal activities. *Afr. J. Microbiol. Res.* **21**, 2243–2248.

Liu, J.Y., Song, Y.C., Zhang, Z., Wang, L., Guo, Z.J., Zou, W.X., and Tan, R.X. 2004. *Aspergillus fumigatus* CY018, an endophytic fungus in *Cynodon dactylon* as a versatile producer of new and bioactive metabolites. *J. Biot.* **114**, 279–287.

Mourad, M.H.E.S. 2010. New bioactive compounds from *Verticillium alboatrum* and *Verticillium leptobactrum*. *Aust. J. Basic Appl. Sci.* **4**, 2166–2175.

Ninthya, K. and Muthumary, J. 2011. Bioactive metabolites produced by *Phomopsis* sp. an endophytic fungus in *Allamanda cathartica* Linn. *Recent Res. Sci. Technol.* **3**, 44–48.

Oyama, M. and Kubota, K. 1993. Induction of antibiotic production by protease in *Bacillus brevis* (ATCC8185). *J. Biochem.* **113**, 637–641.

Qiu, Z.Q., Cao, L.X., Tan, H.M., and Zhou, S.M. 2006. Isolation and characterization of endophytic *Stretomyces* sp. S5 with herbicidal activity from tomato roots. *Chin. J. Agr. Biotechnol.* **3**, 7–12.

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J., and Kirtikara, K. 2007. Metabolites from endophytic fungus *Phomopsis* sp. PSU-D15. *Phytochem.* **69**, 783–787.

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

Sreekanth, D., Sushim, G.K., Syed, A., Khan, B.M., and Ahmad, A. 2011. Molecular and morphological characterization of a taxol-producing endophytic fungus, *Gliocladium* sp. from *Taxus baccata*. *Mycobiology* **39**, 151–157.

Strobel, G.A. 2003. Endophytes as sources of bioactive products. *Microb. Infect.* **5**, 535–544.

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

Tansuwan, S., Pornpakakul, S., Roengsumran, S., Petsom, A., Muangsin, N., Sihanomta, P., and Chaichit, N. 2007. Antimalarial benzoquinone from an endophytic fungus *Xylaria* sp. *J. Nat. Prod.* **70**, 1620–1623.

Tong, W.Y., Darah, I., and Latiffah, Z. 2011. Antimicrobial activities of endophytic fungal isolates from medicinal herb *Orthosiphon stamineus* Benth. *J. Med. Plants Res.* **5**, 831–836.

Valgas, C., Souza, S.M., Smania, E.F.A., and Smania, A.J. 2007. Screening methods to determine antibacterial activity of natural products. *Braz. J. Microbiol.* **38**, 369–380.

Weber, R.W., Kappe, R., Paululat, T., Moskev, E., and Anke, H. 2007. Anti-candida metabolites from endophytic fungi. *Phytochem.* **68**, 886–892.

Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., Sun, P., and Qin, L. 2010. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiol. Res.* **165**, 437–449.