EFFECT OF VOLATILE ORGANIC COMPOUNDS FROM *PHOMOPSIS* SP. (JA1B1-2) AS PLANT GROWTH PROMOTERS AND THEIR ANTIFUNGAL ACTIVITIES

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ABSTRACT

A volatile metabolite-producing endophytic fungus, *Phomopsis* sp. (JA1B1-2) was isolated from leaves of *Jatropha podagria* (Hook) in northern Thailand by a parallel-growth isolation technique and tested for antagonism against phytopathogenic fungi using dual culture assay. The results showed that *Phomopsis* sp. (JA1B1-2) inhibits the mycelial growth of *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Phellinus noxius*, *Phytophthora parasitica*, *Rhizoctonia solani* AG-2 and *Rigidoporus microporus* with between 60.4±2% and 82.1±1% inhibition. The fungus achieved this by producing a mixture of volatile organic compounds (VOCs) with both antifungal and plant growth promoting properties. The parallel-growth isolation technique was adapted for the antagonism test of the VOCs produced by *Phomopsis* sp. (JA1B1-2) and the results showed that they are active against *Aspergillus flavus, A. niger, C. gloeosporioides, F. oxysporum* f. sp. *vasinfectum, Rh. solani* AG-2 and *R. microporus* with between 38.2±6% and 21.8±2% inhibition but have no effect on *Ph. noxius* and *P. parasitica*. The allelochemical effect of VOCs were tested on roots of two dicots, tomato (*Lycopersicon esculentum* Mill.) and mung bean (*Vigna radiata* L.) and two monocot, jasmine rice (*Oryza sativa* L.) and sweet corn (*Zea mays* L. var *saccharata*). They promoted root growth in tomato and jasmine rice. The preliminary measurements of indole-3-acetic acid (IAA) and siderophore production were tested. *Phomopsis* sp. (JA1B1-2) produced IAA 14.7±2 μg/ml and produced siderophore on chrome azurol S (CAS) agar plates.

1. INTRODUCTION

The genus *Phomopsis* (Sacc.) Bubák is a fungus that belongs to the family *Valsaceae*. It contains a large number of plant pathogens which cause blight, canker, and dieback, rot spot and wilt in economic plants (Janse van Rensburg, et al., 2006). However, it is also prevalent as endophytic fungi of many hosts in temperate and tropical regions and often found in the sapwood of angiosperms (Boddy & Griffith, 1989; Udayanga, et al., 2011) *Phomopsis* is a rich source of many bioactive secondary metabolites including antimalarial phomoxanthones (Isaka, et al., 2001) and antifungal phomoxanthone A (Elsaesser, et al., 2005). *Phomopsis* sp. isolated from the leaves of *Vitex negundo* L. showed significant antimicrobial activity against human pathogenic bacteria (Desale & Bodhankar, 2013). Furthermore, the previous report has shown that *Phomopsis* is an interesting bioactive gas-producing endophyte that produced a mixture of volatile compounds such as “sabinene”, a primary substance was not previously known from fungi and has fuel potential (Singh, et al., 2011), including 3-methyl-1-butanol, a volatile antimicrobial metabolite produce by endophytic fungi from the genus *Muscodor* (Siri-udom, et al., 2015). In addition, VOCs produced by *Phomopsis* has biological activity against a wide range of fungal pathogens such as *Pythium, Phytophthora, Sclerotinia, Rhizoctonia, Fusarium, Botrtis, Verticillium* and *Collectotrichum* (Singh, et al., 2011).

In this study, we aimed to evaluate the effective of volatile organic compounds produce by an endophytic fungus, giving a high priority to the genus *Phomopsis*. Antifungal activities and plant growth promoting properties of VOCs from endophytic fungus were tested.

2. EXPERIMENT

2.1 Fungal isolation and storage

The fungal endophyte culture (JP1B1-2) was isolated

from branch of Thai medicinal plant, *Jatropha podagrica* Hook in Chiang Mai province. Plant materials were prepared and surface sterilized following the procedure described by Suwannarach, et al. (2010). A parallel-growth isolation technique was used for isolation of volatile-producing endophytic fungi (Worapong, et al., 2001). A bioactive gas-producing endophytic fungi, *Muscodor heveae* (MB809310) (Siri-Udom, et al., 2015) was used as a reference strain for this isolation. The hyphal tips of fungal endophyte that grew out from the plant segments were aseptically transferred to a fresh PDA plate. The pure cultures were subsequently stored in 20% (v/v) glycerol at -20 °C and deposited at the Sustainable Development of Biological Resources (SDBR) Lab, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, and the BIOTEC Culture Collection (BCC), Bangkok, Thailand.

2.2 Phenotypic classification

Culture characteristics of endophytic fungus were observed with a light microscope (Olympus CH30, Japan).

2.3 Genotypic classification

A pure culture of fungal endophyte (JP1B1-2) was grown on PDA at 25±2 °C for 5 day. The aerial mycelium was scraped from the PDA surface, then freeze-dried and ground into a fine powder with a pestle and mortar. A modified SDS-CTAB method (Suwannarach, et al., 2013) was used for the DNA extraction. The PCR procedure following the procedure described by Siri-Udom, et al. (2015) using a pair of universal primers (ITS4 and ITS5). The purified PCR product was then sequenced by 1st Base, Malaysia. Phylogenetic analysis of all sequences was conducted by the neighbor-joining method using MEGA6 software (Tamura, et al., 2013).

2.4 Antifungal assay

The dual culture technique was used for hyphae growth inhibition of phytopathogenic fungi (Yurnaliza, et al., 2014). While, the parallel-growth isolation technique was adapted for the antagonism test of fungal VOCs (Strobel, et al., 2001). The percent inhibition of fungal growth was calculated with the following equation: $[(R\,1 - R\,2) \times 100] \div R\,1$, where R 1 is the average colony radius of each fungal pathogens measured in the control plates without the fungal endophyte culture (JP1B1-2), and R 2 is the average colony radius calculated from the test plates.

2.5 Allelochemical effect test of the VOCs

Two dicots, tomato (*Lycopersicon esculentum* Mill.) and mung bean (*Vigna radiata* L.), and three monocot, jasmine rice (*Oryza sativa* L.), sweet corn (*Zea mays* L. var *saccharata*) and ruzi grass (*Brachiaria ruziziensis*) were used for this study. The bioassays following the method described by Macías-Rubalcava, et al. (2010). The Petri dishes were divided into two compartment containing PDA and 1% water agar in the opposite side. An agar plug (6-mm diameter) of fungal endophyte (JP1B1-2) was inoculated on PDA compartment and incubated at 30°C for 2 day before introducing seeds into the other compartment. All Petri dishes were wrapped with two layers of parafilm® M and placed in a growth chamber at 28°C and a photoperiod of 12:12h fluorescent light. Root and shoot lengths were measured after 5 day of exposure to fungal VOCs and compared to controls that did not have fungal endophyte (JP1B1-2).

2.6 Preliminary test of IAA production

A modified IAA production test (Hung and Annapurna, 2004) was used in this study. An agar plug of fungal endophyte (JP1B1-2) was inoculated in 5 ml of PDB amended with 0.2% L-tryptophan. The test tubes were covered with aluminium foil and incubated at 25±2 °C for 5 day on the shaker. The culture broth was collected and added with Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 49 ml of 35% perchloric acid ($HClO_4$)) (1:2 v/v), then incubated at 25±2 °C for 30 min. Their absorbance was read at 530 nm using a spectrophotometer compared with IAA standard (10-250 μg/ml). The amount of IAA was estimated using a standard curve.

2.7 Preliminary test of siderophore production

An agar plug of fungal endophyte (JP1B1-2) was inoculated on to CAS-substrates with modified Gaus No. 1 (MGs-1) medium (You et al., 2004) and incubated at 25±2 °C for 5 day. The presence of siderophore is indicated by decolourization of the blue coloured ferric-dye complex, resulting in a yellow, orange or pink halo around the colonies (Srivastava, et al., 2013).

3. ANALYSIS

3.1 Morphology of endophytic culture (JA1B1-2)

The endophytic fungi culture (JA1B1-2) was classified morphologically into the genus *Phomopsis* and closed to *P. longicola* supported by 95% similarity of the gene sequence (Fig. 1). The morphological characteristics fit the description of genus *Phomopsis* (Cui, et al., 2009; Singh, et. al., 2011). The isolate was cultured on PDA and colony was cottony and white with occasional olive-gray areas. The underside of the colony was colorless. Mycelium form thick-rope like structures. It produced hyaline and filiform beta-conidia.

3.2 Antifungal assay

The antagonism test showed that *Phomopsis* sp. (JA1B1-2) has fast growth rate and able to inhibits the mycelial growth of *C. gloeosporioides, F. oxysporum* f. sp *vasinfectum*, *Ph. noxius*, *P. parasitica*, *Rh. solani* AG-2 and *R. microporus* with between 60.4±2% and 82.1±1% inhibition (Table 1) (Fig. 2). The effect of VOCs produced by *Phomopsis* sp. (JA1B1-2) were lethal to *C. gloeosporioides, F. oxysporum* f. sp. *vasinfectum*, *Rh. solani* AG-2 and *R. microporus* with between 21.8±2% and 38.2±6% inhibition (Table 1) (Fig. 2). The previous study showed that VOCs from *Phomopsis* sp. against a broad of fungal pathogens including *Rh. solani* (Singh, et al., 2011).

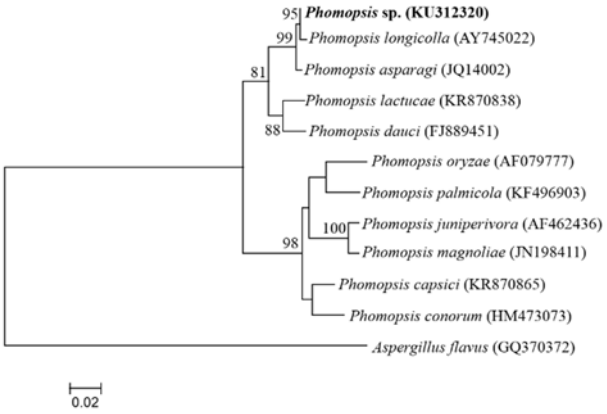

Fig. 1 Maximum parsimony tree of *Phomopsis* sp. based on ITS1-5.8S rDNA-ITS2 sequence alignment of 12 sequences. *Aspergillus flavus* was used as an out group.

Table 1 Antifungal activity of *Phomopsis* sp. (JA1B1-2).

Test organisms	Percent inhibition of mycelial growth	
	Non-volatile	Volatile
Colletotrichum gloeosporioides	60.4±2	21.8±2
Fusarium oxysporum f. sp. *vasinfectum*	71.4±3	26.7±1
Phellinus noxius	81.5±2	0
Phytophthora parasitica	80.8±2	0
Rhizoctonia solani AG-2	65.9±3	35.4±4
Rigidoporus microporus	82.1±1	38.2±6

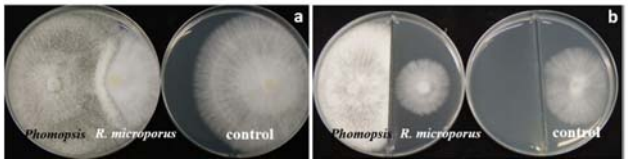

Fig. 2 Antagonism test of *Phomopsis* sp. (JA1B1-2) against mycelial growth of *R. microporus*; fast growth rate more than the pathogen (a), VOCs inhibit the pathogen (b).

3.3 Allelochemical effect test of the VOCs

Allelopathy of microorganism is often demonstrated by testing allelochemical effects on seed germination. There are no effect of VOCs produced by *Phomopsis* sp. (JA1B1-2) to mung bean and sweet corn germination. However, VOCs could promote root elongation in tomato and jasmine rice (Table 2) (Fig. 3). *Phomopsis* sp., an endophytic fungi of *Odontoglossum* sp. produced a mixture of volatile metabolites including sabinene that only known from higher plant but not have function to promote plant growth (Singh, et al., 2011). There are some plant growth-promoting rhizobacteria (PGPR) produced VOCs that promote growth *of Arabidopsis thaliana* (Ryu, et al., 2003). This is first study that VOCs produced by *Phomopsis* has plant gowth promoting activities.

Table 2 Plant growth promoting activity of VOCs from *Phomopsis* sp. (JA1B1-2).

Plant	*Phomopsis*		Control	
	Root (cm)	Shoot (cm)	Root (cm)	Shoot (cm)
Tomato	10.5±2	3.8±1	4.8±2	2.6±1
Mung bean	6.7±1	4.7±1	7.1±1	4.2±1
Sweet corn	8.9±3	5.3±2	8.2±2	6.8±2
Jasmine rice	3.8±1	2.7±1	2.7±1	2.6±1

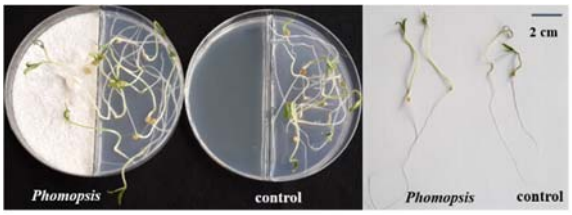

Fig. 3 Plant growth promoting activity of VOCs from *Phomopsis* sp. (JA1B1-2) for 5 days on the root length of tomato.

3.4 Preliminary test of IAA and siderophore production

Many studies have showed that some plant-associated fungi produced IAA (Ludwing-Müller, 2004), the common natural auxin (plant hormone) that affects plants's physiology such as the differentiation of phloem and xylem, and the development of branch roots (Kelen, et al., 2004). The results showed that *Phomopsis* sp. (JA1B1-2) produced IAA 14.7±2 μg/ml. Furthermore, it produced siderophore on chrome azurol S (CAS) agar plates with pink halo around the colonies. Fungi produce and secrete siderophores into the soil to chelate or bind tightly iron and uptake into the cell. Then, fungi will use iron for organism's survival (Winkelmann, 2007).

CONCLUSION

An endophytic fungus, *Phomopsis* sp. (JA1B1-2) was produced VOCs with antifungal activity and showed plant growth promoting activities in tomato and jasmine rice. The mixture of volatile compounds will be identified by a modified gas chromatography-mass spectrometry (GC-MS), including the plant growth promoting activities will be analyzed. IAA will be extracted by TLC, purified and detected. Chemical nature of siderophore will be detected.

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REFERENCES

Boddy, L. and Griffith, G. S. 1989. Role of endophytes and latent invasion in the development of decay communities in sapwood of angiospermous trees. *Sydowia*, vol. 41, pp. 41-73.

Cui, Y. L., Duan, C. X., Wang, X. M., Li, H. J. and Zhu, Z. D. 2009. First report of *Phomopsis longicolla* causing soybean stem blight in China. *Plant Pathol.*, vol. 58, p. 799-799.

Desale, M. G. and Bodhankar, M. G. 2013. Antimicrobial activity of endophytic fungi isolated from *Vitex negundo* Linn. *Int. J. Curr. Microbiol. App. Sci*., vol. 2, pp. 389-395.

Elsaesser, B., Krohn, K., Floerke, U., Root, N., Aust H., Draeger, S., Schulz, B., Antus, S. and Kurtan, T. 2005. X-ray structure determination, absolute configuration and biological activity of phomoxanthone A. *Eur. J. Org. Chem.*, vol. 21, pp.4563-4570.

Hung, P. Q. and Annapurna, K. 2004. Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). *Omonrice*, vol. 12, pp. 92-101.

Isaka, M., Jaturapat, A., Rukseree, K., Danwisetkanjana, K., Tanticharoen, M., Thebtaranonth, Y. 2001. Phomoxanthones A and B, novel xanthone dimmers from the endophytic fungus *Phomopsis* species. *J. Nat. Prod.*, vol. 64, pp. 1015-1018.

Janse van Rensburg, J. C., Lamprecht, S. C., Groenewald, J. Z., Castlebury, L. A. and Crous, P. W. Characterisation of *Phomopsis* spp. associated with die-back of rooibos (*Aspalathus linearis*) in South Africa. *Stud. Mycol*., vol. 55, pp. 65-74.

Kelen, M., Çubuk Demiralay, E., Sen, S. and Özkan, G. 2004. Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99R (*Vitis berlandieri* × *Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) by reverse phase liquid chromatography. *Turk. J. Chem*., vol. 28, pp. 603-610.

Ludwig-Müller, J. 2004. From auxin homeostasis to understanding plant pathogen and plant symbiont interaction: Editor's research interests. *J. Plant Growth Regul.*, vol. 23, pp. 1-8.

Macías-Rubalcava, M. L., Hernández-Bautista, B. E., Oropeza, F., Duarte, G., González, M. C., Glenn, A. E., Hanlin, R. T. and Anaya, A. L. 2010. Allelochemical effects of volatile compounds and organic extracts from *Muscodor yucatanensis*, a tropical endophytic fungus from *Bursera simaruba*. *J. Chem. Ecol*., vol. 36, pp. 1122-1131.

Ryu, C., Farag, M. A., Hu, C., Reddy, M. S., Wei, H., Paré, P. W. and Kloepper, J. W. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Plant Bio.*, vol. 100, pp. 4927-4932.

Singh, S. K., Strobel, G. A., Knighton, B., Geary, B., Sears, J. and Ezra, D. 2011. An endophytic *Phomopsis* sp. possessing bioactivity and fuel potential with its volatile organic compounds. *Microb. Ecol*., vol. 61, pp. 729-739.

Siri-Udom, S., Suwannarach, N. and Lumyong, S. 2015. Existence of *Muscodor vitigenus*, *M. equiseti* and *M. heveae* sp. nov. in leaves of the rubber tree (*Hevea brasiliensis* Müll.Arg.), and their biocontrol potential. *Ann. Microbiol*., DOI 10.1007/s13213-015-1126-x.

Srivastava, M. P., Tiwari, R. and Sharma, N. 2013. Effect of different cultural variables on siderophores produced by *Trichoderma* spp. *IJAR*., vol. 1, pp. 1-6.

Strobel, G. A., Dirkse, E., Sears, J. and Markworth, C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. *Microbiology*, vol. 147, pp. 2943-2950.

Suwannarach, N., Bussaban, B., Hyde, K. D. and Lumyong, S. 2010. *Muscodor cinnamomi*, a new endophytic species from *Cinnamomum bejolghota*. *Mycotaxon*, vol. 114, pp. 15-23.

Suwannarach, N., Kumla, J., Bussaban, B., Hyde, K. D., Matsui, K. and Lumyong, S. 2013. Molecular and morphological evidence support four new species in the genus *Muscodor* from northern Thailand. *Ann. Microbiol*., vol. 63, pp. 1341-1351.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*., vol. 30, pp.2725-2729.

Udayanga, D., Liu, X., McKenzie, E. H. C., Chukeatirote, E., Bahkali, A. H. A. and Hyde, K. D. 2011. The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. *Fungal Divers*., vol. 50, pp. 189-225.

Winkelmann, G. 2007. Ecology of siderophores with special reference to the fungi. *Biometals*, vol. 20, pp. 379-392.

Worapong, J., Strobel, G. A., Ford, E. J., Li, J. Y., Baird, G. and Hess, W. M. 2001. *Muscodor albus* anam. nov., an endophyte from *Cinnamomum zeylanicum*. *Mycotaxon*, vol. 79, pp. 67-79.

You, J. L., Cao, L. X., Liu, G. F., Zhou, S. N., Tan, H. M. and Lin, Y. C. 2004. Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments. *World J. Microbiol. Biotechnol*., vol. 21, pp. 679-682.

Yurnaliza, Aryantha, I. N. P., Esyanti, R. R. and Susanto, A. 2014. Antagonistic activity assessment of fungal endophytes from oil palm tissue againt *Ganoderma boninense* Pat. *Plant Pathol. J*., vol. 13, pp. 257-267.

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