Proceedings of International Conference on Biodiversity: IBD2019 (2019); 9 - 15

Endophytic fungi from wild plants and their antifungal and plant-growth promoting properties

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Abstract: A total of 118 endophytic fungi were isolated from 21 wild plants in northeastern of Thailand. Most endophyte were isolated from *Maesa ramentacea* (Roxb.) A. DC. (12.7%), followed by *Baeckea frutescens* (11.9%) and *Anneslea fragrans* Wall. (6.8%), respectively. Endophytic fungi were more prevalent in the leaves (59.3%) than the branches/stems and the flowers. Isolate Gu02 and Gu03 from flowers of *Gluta usitata* (Wall.) Ding Hou exhibited board range of antifungal activity above 50% growth inhibition by dual culture technique toward to *Colletotrichum gloeosporioides*, *Phellinus noxius* and *Rigidoporus microporus*. Isolate Gu03 inhibited mycelial growth of the pathogenic fungi, *P. noxius* and *R. microporus* causing root rot disease of rubber tree with 74.1% and 88.1% inhibition, respectively. Isolate Gu03 produced plant growth hormone, indole acetic acid (IAA) at the concentration of 331.5 µg/ml. Volatile metabolite-producing endophytic fungus, isolate Cs05 was isolated from leaf tissues of *Cycas siamensis* Miq. The volatile organic compounds (VOCs) of isolate Cs05 were active against *P. noxius* and *R. microporus* with 100% growth inhibition.

Keywords: Antifungal activity, endophytic fungi, indole acetic acid, wild plant

Introduction

Endophytic fungi live in symbiosis with plant tissues and play an important role in plant growth (Young-Hyun et al., 2012). They have been identified in nearly 300,000 plant species (Strobel and Daisy, 2003) and dwell in root, stem, leaf, flower, fruit and seed (Yu et al., 2018). Most endophytic fungi are members of the Ascomycota, with only a few reports of basidiomycetous endophytes, these often being orchid mycorrhizas (Rungjindamai et al., 2008). Natural select the evolution of beneficial endophyte strains and several endophytes are found to produce bioactive compounds that protect plant from insect pests and pathogens (Saikkonen et al., 2004). They play an important role in plant defense including the function as growth promoter and enable the host survival under extreme conditions (Rosa et al., 2012). Although, chemical pesticides were used to protect plant disease, but pesticide use may lead to environmental pollution and might threaten human health (Yu et al., 2018). Recently, the development and spread of drug-resistant pathogens are still a global problem and there is a need to search for new active agents with antimicrobial activity (Espinel et al., 2001). Thus, endophytic fungi are source of novel or bioactive metabolites for pharmacological and agricultural applications (Idris et al., 2013; Deshmukh et al., 2018).

Natural products from fungal endophyte showed antagonistic activity to inhibit several pathogenetic organism such as bacteria, fungi, viruses and protozoans. Furthermore, they could be plant growth regulators such as indole acetic acid (IAA), one of the most physiologically active auxins. Fungi produce a wide variety of plant hormone such as gibberellins (GAs), abscisic acid (ABA), and auxin (Young-Hyun et al., 2012; Khan et al., 2017). IAA is a product of L-tryptophan metabolism by various microorganism including Plant growth promoting rhizobacteria (PGPR) (Ahmad et al., 2005). The volatile metabolite-producing endophytic fungi have not been commonly reported. But some endophytic fungi that belong to the families Diaporthaceae, Hypocreaceae, Stachybotryaceae and Xylariaceae of the phylum Ascomycota are notable for their capacity to form volatile metabolites with antimicrobial activity (Stinson et al., 2003; Banerjee et al., 2010; Suwannarach et al., 2013; Siri-Udom et al., 2015). The mixture of fungal volatile compounds that have been identified were aldehydes, alcohols, benzene derivatives, cyclohexanes, ketones, hydrocabons, heterocycles, phenol, thioalcohols, thioesters and their derivatives (Mercier et al., 2007; Morath et al., 2012). In agriculture, fungal VOCs with antimicrobial activity may be used as a mycofumigant to control plant disease.

In this study, we aimed to evaluate the diversity of endophytic fungi that live in association with wild plants. Furthermore, to assessed the potential of the effective fungi that capable of producing bioactive agents, plant growth promoting metabolites and volatile metabolites against fungal pathogens.

Materials and Methods

Preparation and surface sterilization of plant materials

Twenty-one healthy wild plant species were used as source for isolation of endophytic fungi. Eight plants were collected from The Royal-initiated Lam Huai Bong Forest Area Development Project (16°54′23″N, 102° 24′47″E), located in, Nong Bua Lam Phu Province. Six plants were collected in Dong Kheng community forestry, Nong Bua Lam Phu Province (16°56′49″N, 102°34′31″E). Seven plants were collected from Ban Thai Seri community forestry, Bueng Kan Province (18°00 ′54″N, 103°57′49″E). Plant materials were cut randomly into small segments (leaf and flower, 5 mm x 5 mm; stem/branch 5 mm long). All segments were sterilized by soaking in 75% ethanol for 30 s, 2% sodium hypochlorite for 3 min, and 95% ethanol for 30s under a laminar flow hood (Suwannarach et al., 2010).

Isolation of endophytic fungi

Each plant materials were placed on potato dextrose agar (PDA) containing Rose bengal (0.033 g/l) and chloramphenicol (50 mg/l). Petri dishes were sealed with Parafilm® M (Bemis company, Inc., USA) and incubated at room temperature (25±2 °C) for 2 weeks. Emerging fungi were transferred to fresh PDA plates. The medium was also be used for subculture and stock culture. Identification of endophytic fungi was carried out on the basis of morphological characteristics (Huang et al., 2008). The fungal isolates were grown on PDA and incubated at room temperature to observe morphological characteristic such as color, shape and size of spores. Spore production of endophytic fungi was studied on different media, such as malt agar (MA), PDA and water agar (WA).

Antagonism against fungal pathogens by dual culture method

The antagonistic analysis was observed from the interaction between fungal endophytes and the pathogens using dual culture technique. An agar plug (6 mm diameter) 4-day-old fungal endophytes growing on PDA was inoculated on PDA part of the petri dish, then an agar plug of fungal pathogens was inoculated opposite side of the Petri dish. The control plates were inoculated with either pathogens. All Petri dishes were wrapped with Parafilm® M and incubated at room temperature (25±2 °C) for 7 days. The percentage of inhibition of fungal growth after the dual culture test was calculated with the following equation: $[(R1- R2)\times100] \div R1$, where R 1 was the average colony radius of each tested fungi measured in the control plates (without the tested fungi), and R 2 was the average colony radius that calculated from the tested plates.

Antimicrobial assay of VOCs

The parallel-growth isolation technique was adapted for the antagonism test of fungal VOCs. An agar plug of volatile-producing endophytic fungi was inoculated on the PDA part of a two-compartment Petri dish, and allowed to grow at room temperature (25±2 °C) for 2-4 days. Then, an agar plug (6-mm diameter) 4-day-old fungal pathogens growing on PDA was inoculated on PDA on the opposite side of the Petri dish. All Petri dishes were wrapped with parafilm® M and incubated at room temperature (25±2°C) for 3-7 days. The percent inhibition of fungal growth after the dual culture test was calculated. The viability of the test fungal pathogens was observed by transferring them from the test plates and re-growing in fresh PDA (Strobel et al. 2001).

Indole acetic acid (IAA) production

All isolates of endophytic fungi were inoculated in 5 ml potato dextrose broth (PDB) with L-tryptophan (2 mg/ml) and incubated in the dark at room temperature (25±2 °C) with shaking at 150 rpm on a shaker for 5 days. The broth cultures were filtrated by two layers of gauze cloth to separate the broth cultures and mycelia. A modified method described by Ahmad et al., 2005 was used for screening IAA production. All filtrates (1 ml) were mixed with 2 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$; 50 ml of 35% Perchloric acid ($HClO_4$)) and incubated in the dark for 30 min. The development of pink color indicated IAA production and the absorbance at 530 nm was measured. The level of IAA production was estimated by standard IAA graph.

Result & Discussion

Isolation of endophytic fungi

A total of 118 endophytic fungi were isolated from 21 plant species, the most of them were isolated from *Maesa ramentacea* (Roxb.) A. DC. (12.7%), followed by *Baeckea frutescens* (11.9%) and *Anneslea fragrans* Wall. (6.8%), respectively (Table 1). Most of them colonized in leaf (59.3%) and only 3 isolates colonized in flower of plant sample (Figure 1. and Table 1). Furthermore, 6 volatile producing endophytic fungi were isolated from 5 wild plants, including *M. ramentacea*. Isolate Cs05 did not produce spore and belonged to mycelia sterilia group. Endophytic fungi are presented in most of plant section, especially healthy leaf tissue (Kharwar et al., 2011; Karunai and Balagengatharathilagam, 2014; Yu et al., 2018) according to this study, most endophytic fungi were more prevalent in the leaves than the branches/stems and the flower. Although, Zheng et al. (2016) proposed that the diversity of endophytic fungi is generally significantly higher in the stems than in the leaves.

Endophytic population varies from plants to plants and species to species (Nair and Padmavathy, 2014). However, the endophytes population was affected by the environmental conditions under which the host is growing and the endophyte profile may be is more diversified in tropical areas (Karunai and Balagengatharathilagam, 2014). The isolate numbers of endophytic fungi are closely correlated with the sampling range of the plant age, such as increasing the collecting plant specimens of different ages, to enable the isolation of additional fungal endophytes (Yu et al., 2018).

Wild plant in family Myrsinaceae can potentially be explored for estrogenic activity and for sources of phytoestrogens (Jamal et al., 2012), genus *Maesa* was reported the fungal endophyte in genus *Diaporthe* lived in fruit (Gomes et al., 2013). *Coniochaeta ligniaria*, an endophytic fungus with antifungal activity was isolated from leaves of *Baeckea frutescens* (family Myrtaceae) obtained from the Phu Luang wildlife sanctuary in Thailand (Kokaew et al., 2011). Fungal endophyte can live in various wild plant tissue, genus *Fusarium* was the most common fungal isolate from root of wild banana (*Musa acuminate*, family Musaceae), which is native to Southeast Asia and is an ancestor of the edible banana (Zakaria et al., 2016). Several endophytic fungi, such as *Colletotrichum gloeosporioide, Phomopsis* sp., *Phyllosticta capitalensis* and *Corynespora* were consistently isolated from the different tree in tropical forest of southern India. In dry season, endophytic diversity was greater in the dry thorn forest than in the dry deciduous forest (Murali et al., 2007).

Antagonistic activity of endophytic fungi

Based on the results, 22 isolates of endophytic fungi could inhibit the mycelial growth of fungal pathogens and only 16 isolates showed the percentage of inhibition above 50%. Isolate Gu03 showed the greatest percentage of inhibition of mycelial growth with *R. microporus* (88.1%) and *P. noxius* (74.1%), respectively (Figure 2. and Table 2). Isolate Cc12 showed the greatest percentage of inhibition of mycelial growth with *C. gloeosporioides* (64.9%) by overgrowth of the tested pathogen (Table 2).

Root rot disease is the most serious problem of rubber tree plantation and present in many countries, including Thailand. In this study, the antagonistic test showed that the causing pathogen, *R. microporus* and *P. noxius* was against by isolate Gu02 and Gu03 with the highest percentage of inhibition. The antagonistic activity of fungi to inhibit mycelial growth of the root rot pathogens was considered to control root rot disease in the field according to the previous studies such as the use of *Trichoderma hazianum* (Jayasuriya and Thennakoon, 2007) and *Chaetomium cupreum* to control *R. microporus* in vivo (Kaewchai and Soytong, 2010).

The bioactive compounds were produced by the plant but in a mutualistic association with the host plant, endophytes may enhance bioactive metabolites that process bioactivity such as antibacterial and antifungal activity in host plant (Radu and Kqueen, 2002; Tejesvi et al., 2007).

Antimicrobial assay of VOCs

The volatile metabolites produce by endophytic fungi were tested for their antimicrobial activity. Isolate Cs05 showed the greatest antimicrobial activity with all test organisms (Table 3). It was isolated from leaf tissues of *Cycas siamensis* (family Cycadaceae), which did not produce spores on several media, including MA, PDA and WA, and had rope-like mycelium with coiled structures. Many endophytic fungi are known to produce the bioactive compounds in the form of volatile metabolites with anti-microbial, anti-oxidant and anti-proliferative activities, cytotoxicity, and fuel production (Naik, 2018). In the family Xylariaceae, Muscodor albus is the first known fungal endophyte isolated from *Cinnamomum zeylanicum*, which produces bioactive volatile metabolites (Strobel et al., 2001; Ezra et al., 2004). An endophytic fungus, *Nodulisporium* sp. produced the mixture of volatile compounds were active against plant pathogens. The most abundant identified compound was 1, 8 cineole, 1-butanol, 2-methyl, and phenyl ethanol alcohol and most importantly cyclohexane, propyl, which is a common ingredient of diesel fuel (Hassan et al., 2013).

Indole acetic acid (IAA) production

Eighteen isolates of endophytic fungi showed their ability to produce IAA in preliminary test. The range of IAA production was 11.3 µg/ml to 331.5 µg/ml (Table 2). Endophytic fungus, isolate Gu03 produced maximum concentration of IAA, following by isolate Gu01 and Cc12, respectively (Table 2). This result was supported by the previous studies that showed several endophytic fungi, which produced IAA and increased seed germination and plant growth (Khan et al., 2012, 2015, 2017; Zhou et al., 2013; Kedar et al., 2014). Endophytic fungi enhance plant growth by produce various secondary metabolites, including IAA, flavonoids and flavnols. These compounds were determined in the culture filtrate of *Aspergillus fumigatus*, an endophyte in leaves of wild plant in family Solanaceae, Withania somnifera (Mehmood et al., 2018). IAA is essential for crop growth and development because it enhances root, flower development, and other processes (Reinhardt et al., 2000). However, there are reports that IAA is important tool to against fungal pathogens, such as *Colletotrichum* spp. (Yue et al., 2000).

Conclusion

Endophytic fungi colonized in plant tissue, especially healthy leaf tissue. They had antimicrobial and plant-growth promoting properties. Biological control by endophytic fungi to prevent disease and promote plant growth offer an attractive alternative method for disease management without the negative impact of the chemical control. Furthermore, volatile metabolite-producing endophytes may be an alternative biological approach as biofumigation in control of plant diseases.

Acknowledgement

This study was supported by grants from Research and Development Institute Udon Thani Rajabhat University.

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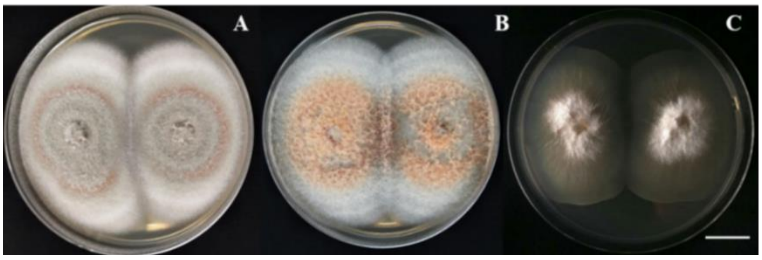

Figure 1. Colony of isolate Gu02 and Gu03 on PDA (A-B) and isolate Cs05, volatile-producing endophytic fungus on PDA (C), scale bar = 2 cm.

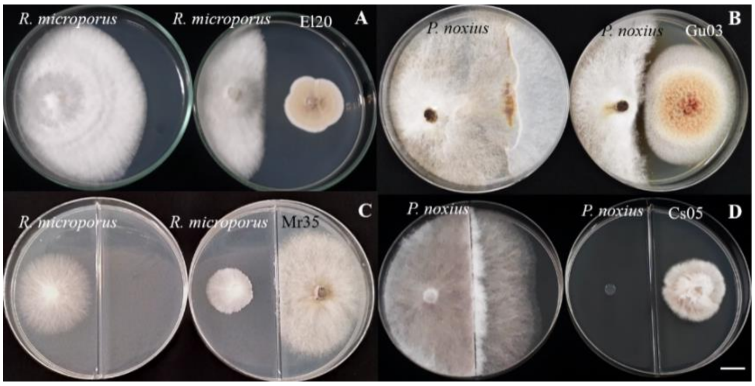

Figure 2. Preliminary testing for antifungal activity of endophytic fungi (isolate El20 and Gu03) against plant pathogenic fungi by dual culture technique (A-B). Antifungal activity of VOCs from isolate Mr35 and Cs05 after the exposure of VOCs for 3 and 7 day, respectively (C-D), scale bar = 1.5 cm.

Table 1. Number of endophytic fungi isolated from wild plants.

Plant species	Family	Leaf		Stem/Branch	Flower	Total
		Vein	Intervein			
** The Royal-initiated Lam Huai Bong Forest Area Development Project*						
Cycas siamensis	Cycadaceae	2	0	1	0	3
Dipterocarpus tuberculatus	Dipterocarpaceae	0	2	2	0	4
Ficus microcarpa	Moraceae	1	1	2	0	4
Gluta usitata	Anacardiaceae	0	0	0	3	3
Hoya ovalifolia	Asclepiadaceae	2	4	0	0	6
Melientha suavis	Opiliaceae	1	2	2	0	5
Shorea obtusa	Dipterocarpaceae	3	0	3	0	6
Shorea siamensis	Dipterocarpaceae	5	0	1	0	6
** Dong Kheng community forestry*						
Cratoxylum formosum	Clusiaceae	1	0	4	0	5
Dialium cochinchinense	Leguminosae - Caesalpinioideae	2	0	0	0	2
Dioecrescis erythroclada	Rubiaceae	0	0	1	0	1
Ellipanthus tomentosus	Connaraceae	3	0	2	0	5
Erythrophleum succirubrum	Leguminosae - Caesalpinioideae	0	0	1	0	1
Streblus asper	Moraceae	6	1	0	0	7
** Ban Thai Seri community forestry*						
Anneslea fragrans	Theaceae	1	2	5	0	8
Baeckea frutescens	Myrtaceae	6	0	8	0	14
Catunaregam tomentosa	Rubiaceae	2	4	0	0	6
Dipterocarpus obtusifolius	Dipterocarpaceae	1	6	0	0	7

Plant species	Family	Leaf		Stem/Branch	Flower	Total
		Vein	Intervein			
Eurycoma longifolia	Simaroubaceae	2	2	0	0	4
Maesa ramentacea	Myrsinaceae	3	3	9	0	15
Syzygium gratum	Myrtaceae	2	0	4	0	6

Table 2. Antifungal activity and IAA production of endophytic fungi from wild plants.

Isolate number	Percentage of inhibition of microbial growth			IAA (µg/ml)
	C. gloeosporioides	*P. noxius*	*R. microporus*	
Af01	64.1	0	0	74.4
Af03	51.3	0	0	84.4
Af08	37.7	0	0	70.1
Af10	51.3	0	0	79.0
Bf09	26.1	67.2	64.3	0
Cc12	64.9	23.5	5.9	150.8
Cs01	53.9	0	0	32.6
Ct01	54.0	0	0	0
Dc01	53.2	0	0	86.8
De03	63.7	23.5	29.4	76.8
El20	52.5	8.5	54.5	11.3
Et09	40.5	10.3	0	103.9
Fb08-1	54.1	0	0	65.2
Gu01	36.5	7.1	44.7	243.1
Gu02	50.2	65.5	38.0	50.0
Gu03	35.4	74.1	88.1	331.5
Mr28	43.1	31.0	57.1	71.3
Mr29	28.6	69.0	28.6	0
Ms12-2	20.0	0	0	86.3
Ss09	20.2	0	0	53.7
Ss35	12.3	0	0	69.1
Su21	52.2	0	0	0

Table 3. Antifungal activity of VOCs from endophytic fungi.

Plant sample	Isolate number	Percentage of inhibition of microbial growth		
		C. gloeosporioides	*P. noxius*	*R. microporus*
Baeckea frutescens	Bf17	23.5	7.7	43.5
Cycas siamensis	*Cs05*	100/D	100/D	100/D
Hoya ovalifolia	Ho21	0	0	30.4
Maesa ramentacea	Mr23	9.1	65.1	26.1
	Mr35	60.9	1.4	58.8
Melientha suavis	Ms01-2	0	0	21.7

*D = Dead