Antagonistic Effect of Endophytic Fungal, *Fusarium* sp. MFLUCC16-1462, Against *Magnaporthe oryzae*

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Abstract

Sixty-six fungal endophytes were isolated from the leaves of *Litsea petiolata* Hook. f. collected in Chiang Rai, Thailand. Crude extracts obtained from macerated endophytic fungi with ethyl acetate were used to screen their antifungal activity against two isolates (THL084 and THL861) of rice blast disease, *Magnaporthe oryzae*. Based on disc diffusion assay, crude extract of *Fusarium* sp. MFLUCC16-1462 exhibited antifungal activity against the THL084 isolate. The extract also greatly reduced the growth of the THL084 isolate. At 96 h after dual cultures with the *Fusarium* sp. MFLUCC16-1462, mycelium growth of THL084 and THL 861 was inhibited 61.96 and 31.74 %, respectively. The chemical composition of the fungal extract was further identified using a gas chromatographic-mass spectrometric method with their retention indices. Seventy constituents representing 95.92 % of the crude extract were identified. Major components were pregeijerene B, callitrin, drimenol, β-calacorene, benzyl acetylacetate and angustione. Antifungal activity of crude extract *Fusarium* sp. MFLUCC16-1462 could be involved to a combination of chemical constituents such as pregeijerene B, callitrin, drimenol, angustione, Z-lanceol acetate, nor-davanone, 1,4-cineole and 1,8-cineole. These active components could damage cellular membrane and leakage inhibiting spore germination, proliferation, and cellular respiration. It was indicated that the endophytic fungus *Fusarium* sp. MFLUCC16-1462 may suggest an evolutionarily important role for endophytic fungi, associating *L. petiolata* leaves, against fungal plant pathogens, and could be used in agricultural management in the future.

Keywords: biological control; blast; rice disease control; endophytic fungi

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1. Introduction

Rice blast caused by *Magnaporthe oryzae* is one of the major diseases in cultivated rice, which consumes as cereal grain by more than one-half of world population. This destructive disease can cause yield loss up to 100 % (Prabhu *et al*., 2009). The pathogenic fungus will attack mostly on leaves, stem, and panicle and present as chlorosis, necrosis and yellow brown leaf spot or lesions. The *M. oryzae* is extensive to threatened rice production from around the world. Because, it directly affects yield dramatically which results in total economic cost up to $203.49 million annually (Nalley *et al*., 2016). Moreover, spore (conidia) of these pathogenic fungi can easily spread through the wind and sometime can travel more than 1-m range (Ou, 1997). In one day, approximately, 2,000-6,000 conidia are produced from single lesion and it can produce up to 14 days. In addition, dispersal of rice and probably through seed transmission can also introduce this fungus to several countries (Long *et al*., 2001).

Many techniques including chemical treatment and biological method have been developed to mitigate the outbreaks of this disease. However, chemical treatment is providing negative effect to environment and not economical friendly. As Wang (2009) reported that the cost of fungicide to control rice blast outbreaks can be as high as $70 ha^{-1}. Thus, in temperate rice production region this disease is one of the costliest rice diseases (Skamnioti and Gurr, 2009). Another effective way to control this disease is through the use of blast-resistant cultivars (Qu *et al*., 2006). Currently, several resistant cultivars are available such as Rongfeng 3A, Minghui 63, Norin 6, Norin 8, Norin 22, and Norin 23 (Qu and Jennings, 1996; Lei *et al*., 2006; Fu *et al*., 2012). However, the breeding for disease resistance cultivars is both difficult and time consuming. Thus, the cost for seeds of resistant cultivar can be extremely high. For instant, in 2015 hybrid seed that released by RiceTec is approximately $237.12/ha (UACES, 2016). More importantly, since *M. oryzae* is highly variable, after few years of resistant cultivars in the field it can be easily overcome by new strain of fungus that often evolve and mutate (Wang and Valent, 2009; Jia *et al*., 2017).

Due to the limitation in chemical treatment and the use of resistant cultivars, biological method by biocontrol could be alternative way to control rice blast disease. In addition, this technique is sustainable and environmentally favorable. From many kinds of biocontrol, endophytic microorganisms are one type of biocontrol and it gets a lot of attention because it can spread easily in host plants via metabolic translocation same as phyto-pathogens (Firakova, 2007; Rai *et al*., 2017). Endophytes are microorganisms that can colonize plant tissues internally and complete their life cycles without causing any disease or apparent infections (Bacon and White, 2000). These plant-associated microorganisms play important roles in plant protection due to their ability in producing plant-growth regulatory, antibacterial, antifungal, antiviral, and

insecticidal compounds (Yuan *et al*., 2009). Interestingly, several factors affect diversity of endophytes, such as geographical location, part of plant, and plant age (Arnold *et al*., 2003). A large degree of diversity of endophytes has been observed in plants of tropical and subtropical regions. In these regions, approximately fifty to one hundred endophytes were found in a single plant (Stone *et al*., 2004). Thus, plants in the upper Amazon countries, as well as, Indonesia, Thailand and Australia are frequently used as sources to isolate endophytes (Mitchell *et al*., 2010). One interesting plant that originates in Asia is *Litsea petiolata* Hook. f. It is classified in family Lauraceae and has a distinctive smell. Several studies reported that volatile secondary metabolites have broad biological effects against bacterial and fungal pathogens (Heal and Parsons, 2002; Kai *et al*., 2009; Cepl *et al*., 2010).

Rice blast disease is continuing to be a major threat to rice grower from around the world. However, successful strategies to manage this disease are still very limited. Since, ability of endophytes in producing potent metabolites, antibacterial, antifungal, antiviral, and insecticidal compounds are well established (Yuan *et al*., 2009; Mitchell *et al*., 2010). Also, the high degree of diversity of endophytes in tropical plants increases the chance of finding novel antimicrobial natural products. More importantly, there is high possibility that endophytic fungus that isolate from *L. petiolata* will be able to produced volatile secondary metabolites and provide antimicrobial activity. Therefore, the objective of this study is to examine antifungal activity of crude extracts of endophytic fungi isolated from *L. petiolata* leaves against *M. oryzae*. The discovery from this study may lead to new bioactive agents against rice blast disease in the future.

2. Materials and Methods

2.1 Fungal endophytes and pathogens

Sixty-six endophytic fungi were isolated from surface sterilized leaves of *L. petiolata* leaves obtained from Mae Chan, Chiang Rai, Thailand in March 2017. The voucher specimen (MFL No. 10006) obtained by morphological identification of this plant was deposited at the Mae Fah Luang Botanical Garden, Chiang Rai, Thailand. Two isolates of fungal pathogen *M. oryzae* including THL084 and THL861 were obtained from the rice blast fungus genetic stock at the National Center for Genetic Engineering and Biotechnology (BIOTEC, Bangkok, Thailand), the Department of Agronomy, Kasetsart University, Bangkok, Thailand. Each fungal isolate was cultured in a petri dish containing rice flour agar medium (RFA; 2.0 % rice flour, 2.0 % agar and 0.2 % yeast extract and 1 mL/s dH_2O) at room temperature.

2.2 Isolation of fungal endophytes

Procedures for isolating fungal endophytes were adopted followed the methodology described by Monggout *et al.* (2017). The leaves were washed with distilled water followed by 75 % ethanol and then sterilized with 1.5 % sodium hypochlorite and

washed with 75 % ethanol for three times. Sterilized tissue segments were placed on to surface of potato dextrose agar (PDA) medium plates and incubated in the dark at 28 ºC for four days. Each endophytic fungal strain was then isolated and purified by hyphal tip isolation on PDA plates without antibiotics and stored at 4 °C. All isolates were deposited and identified with assigned accession numbers by the Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai, Thailand.

2.3 Extraction by ethyl acetate

All pure fungi were further cultured in potato dextrose broth (PDB) at 28 ºC for 30 days. All cultures were macerated with 150 mL ethyl acetate at 28 ºC for three days and subsequently placed in a separating funnel. Ethyl acetate was used to extract the PDB culture broth. The ethyl acetate extracts from the mycelia and PDB culture broth were combined and concentrated by using a vacuum rotary evaporator. The concentrated crude extracts were subsequently stored at -20 °C.

2.4 *In vitro* antifungal activity

Disc diffusion assay was used to test antifungal activity of 66 crude extract against two blast isolates. Both isolates were cultured in RFA media (100 mm plate) for 7 days. The 6 mm filter paper discs (Whatman, no. 3) were impregnated with 0.1 mg/ml of each crude extract. Discs loaded with crude extract were placed onto the surface of culture plates and placed 8 discs per plate. Ethyl acetate and water were used as negative control. All experiments were performed in triplicate. Tested plates were incubated at room temperature for four days before being measured inhibition zone. The diameters of the zones of complete inhibition were measured including the diameter of the disc.

2.5 Dual-culture assay

As the results of disc diffusion assay, *Fusarium* sp. MFLUCC16-1462 exhibited the most potent antifungal activity against isolate THL084. Thus, this endophytic fungus was further analyzed in dual culture assay. The dual culture was performed by placing the 8-mm mycelia discs from the margins of actively growing cultures of isolate THL084 and THL861 and *Fusarium* sp. MFLUCC16-1462 or *Colletotrichum* MFLUCC16-1508 on the surface of PDA plate. Each PDA plate was inoculated with one endophytic fungus and one pathogen and placed them 3 cm apart. Single culture of each pathogen and single pathogen on fungicide (Tricyclazole, 0.1 g in 200 ml of distilled water) was used as negative control and positive control, respectively. This dual culture assay also performed in tri-replicates. The colony radius (R) of *M. oryzae* was measured at 48, 72 and 96 h after dual culture at room temperature. The average radius of each fungal phytopathogen in the treatment was recorded as R_1, and that in the growth control was recorded as R_2. The inhibition percentage of the growth of the fungal pathogen in the endophyte-pathogen antagonism was calculated with formula as mentioned below:

$$\% \text{ inhibition} = [(R_2 - R_1) \div R_2] \times 100$$

2.6 Chemical composition

The chemical composition in crude extract of *Fusarium* sp. MFLUCC16-1462 was analyzed by gas chromatography-mass spectrometry (GC-MS). This instrument consists of gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP model 5973 mass-selective detector and an electron impact (EI) ion source. The analytes were separated on a HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Agilent Technologies, USA). The oven temperature program was started at 60 °C and increased to 200 °C with a rate of 3 °C/min. High pure helium (99.99 %) was used as the carrier gas and set at a constant flow rate of 1 mL/min. The injection port, transfer line, and ion source temperatures were all set at 250 °C. Seventy electron volts of EI were adopted, and the mass range was scanned from 29 to 300 amu in full scan. The injection was set by split mode with a split ratio of 50 : 1. Solvent delay time was set for five min. MSD ChemStation Data analysis was used to process data. All chemical components in crude extract of *Fusarium* sp. MFLUCC16-1462 were identified by comparing with their retention indices, relative to C_9-C_{19} *n*-alkanes, and comparing the obtained mass spectra of the analytes with those of authentic standards in the Wiley and NIST databases. The identified components and their relative peak area percentage were summarized in Table 2.

3. Results and Discussion

Sixty-six fungal endophytes were isolated from healthy leaves of *L. petiolata* and genera of fungal isolates were also characterized by using taxonomically characters including conidia shape, colony color and texture, conidiophore type and growth rate. All strains were deposited at the Center of Excellence in Fungal Research, Mae Fah Luang University, Thailand. Based on their morphology, 66 endophytic fungi were identified in a group of genera *Fusarium, Colletotrichum*, *Nigrospora*, *Aspergillus*, *Agrocybe*, *Ustilago* and *Puccinia*. Most isolated fungi were classified in a group of the genus *Colletotrichum* which was considered as large genera found in nature. Morphology of some fungi including *Fusarium* sp. MFLUCC16-1462, *Colletotrichum* sp. MFLUCC16-1470 and *Colletotrichum* sp. MFLUCC16-1508 are depicted in Fig. 1.

The crude extracts of all fungi were screened for their antifungal activity against two isolates of rice blast pathogenic fungi, *M. oryzae*. Out of 66 crude extracts, *Fusarium* sp. MFLUCC16-1462, exhibited patent *in vitro* antifungal activity against isolate THL084. In disc diffusion assay, the tentative zone of inhibitions of mycelial growth of *M. oryzae* isolate THL084 was found. Similarity, in dual cultures *Fusarium* sp. MFLUCC16-1462 greatly reduced the growth of isolate THL084. As expected, at 96 h after dual cultures, *Fusarium* sp. MFLUCC16-1462, exhibited 61.96 and 31.74 % inhibition against THL084 and THL861, respectively (Table 1). On the other hand, zero inhibition percentage was exhibited in single and dual culture with *Colletotrichum* MFLUCC16-1508. This indicated that *Fusarium* sp. MFLUCC16-1462 produced

active chemical compounds against *M. oryzae* pathogens. Incubation time and crude extract concentrations may influence on pathogen growth. As noticed, increasing of active compounds concentration resulted by solvent evaporation could decrease mycelia growth of pathogen at 96 h. However, % inhibition of pathogen growth was stopped after 96 h because growth of pathogens was reached the margin of the petri dish in controls.

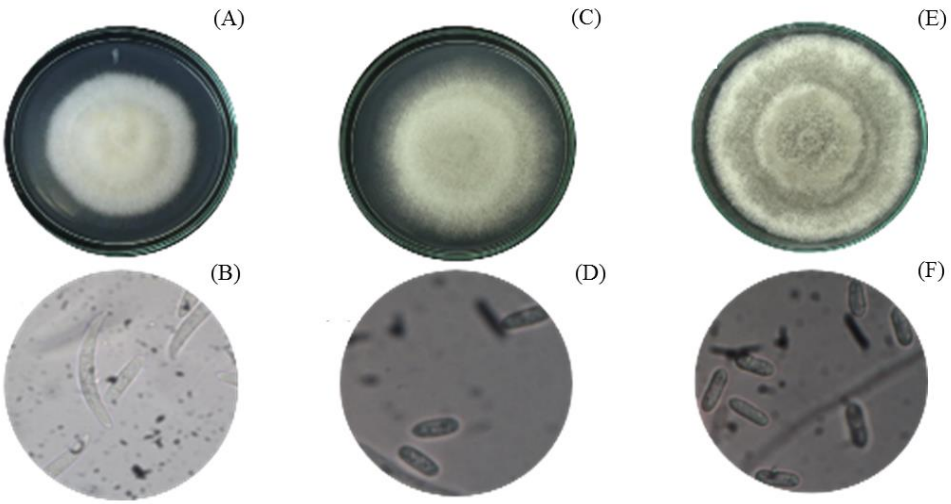

Figure 1 PDA culture of: (A) *Fusarium* sp. MFLUCC16-1462; (B) spores of *Fusarium* sp. MFLUCC16-1462; (C) *Colletotrichum* MFLUCC16-1470; (D) spores of *Colletotrichum* MFLUCC16-1470; (E) *Colletotrichum* MFLUCC16-1508; (F) spores of *Colletotrichum* MFLUCC16-1508 isolated from *L. petiolata* Hook. f. leaves.

Table 1 Percentage inhibition of pathogen growth by fungal endophyte *Fusarium* sp. MFLUCC16-1462

Hour after dual culture	% inhibition		
	Tricyclazole	*M. oryzae* isolate THL084	*M. oryzae* isolate THL861
48	80.68	26.26	15.71
72	78.41	29.19	20.13
96	77.88	61.96	31.74

Due to high antifungal activity against *M. oryzae* of the fungal extract obtained from *Fusarium* sp. MFLUCC16-1462, the chemical composition of crude extract *Fusarium* sp. MFLUCC16-1462 was investigated by gas chromatography-mass spectrometry. All identified compounds with relative peak area percentages and their retention indices are

summarized in Table 2. A total of 70 chemical compounds representing 95.92 % of the crude extract from *Fusarium* sp. MFLUCC16-1462, were identified. Six constituents including pregeijerene B (46.89 %), callitrin (5.51 %), drimenol (4.61 %), β-calacorene (3.32 %), benzyl acetylacetate (2.45 %) and angustione (2.13 %) were considered as major compounds. Benzyl salicylate (1.69 %), methyl tetradecanoate (1.63 %), *Z*-lanceol acetate (1.42 %), iso-menthyl acetate (1.37 %), 2*E*, 4*E*-nonadienol (1.29 %), nor-davanone (1.24 %), hexyl octanoate (1.18 %) and 2*E*,4*E*-decadienal (1.16 %) were also presented as minor constituents. In addition, the antifungal property of *Z*-lanceol acetate, nor-davanone, 1,4-cineole and 1,8-cineole could be also assisted to inhibit *M. oryzae* (Cakin *et al*., 2004; Zengin and Baysal, 2014).

However, bioactive compounds in crude extract of *Fusarium* sp. MFLUCC16-1462 could be contributed to some minor compounds. Chan *et al.* (2016) reported that *Z*-nerolidol, sesquiterpene alcohol, found in the crude extract of *Fusarium* sp. MFLUCC16-1462 contained high pharmacological and biological activities in different *in vitro* and *in vivo* models such as antimicrobial, anti-biofilm, antioxidant, anti-parasitic, skin-penetration enhancer, skin-repellent, anti-nociceptive, anti-inflammatory and anti-cancer. This compound played an important role in the defense system in some medicinal and aromatic plants. Endo-fenchol was considered as an antifungal compound as reported by Sharma *et al*. (2016). Other chemical compounds such as geijerene, o-guaiacol and camphene also evaluated as important fungicide against plant pathogens (Kazuxama, 2017). Various active chemical compounds with different concentration in crude extract could damage cellular membrane and leakage inhibiting spore germination, proliferation, and cellular respiration and resulting fungal cell death in finally (Strobel *et al*., 2002). Since endophytic fungus *Fusarium* sp. MFLUCC16-1462 isolated from *L. petiolate* leaves yields a unique and complexity of biological active components that also have potential antifungal activities relating to the presence of major constituents including pregeijerene B, callitrin, drimenol and angustione, the extract of *Fusarium* sp. MFLUCC16-1462 can be thus considered as bioactive fungus with potential applications in agriculture, forestry, and postharvest.

4. Conclusion

One out of sixty-six endophytic fungi, *Fusarium* sp. MFLUCC16-1462 isolated from the leaves of *Litsea petiolata* Hook. f. showed highest *Magnaporthe oryzae* isolates THL084 and THL861, the causal agent of rice blast disease, mycelium inhibition with 61.96 and 31.74 %, respectively. Moreover, crude extract of *Fusarium* sp. MFLUCC16-1462 exhibited high antifungal activity against rice blast pathogen isolate THL084. Gas chromatographic-mass spectrometric method showed the major components of crude extract of *Fusarium* sp. MFLUCC16-1462 including pregeijerene B, callitrin, drimenol, angustione, *Z*-lanceol acetate,

Table 2 Chemical composition of *Fusarium* sp. MFLUCC16-1462 fungal extract

Compound	RI	% area	Compound	RI	% area
Camphene	942	0.54	phenyl hexan-3-one	1423	0.27
cyclohexyl formate	957	0.38	*p*-acetylacetophenone	1444	0.11
3-octanone	975	0.81	neryl propanoate	1451	0.05
butyl butanoate	993	0.69	cyclamen aldehyde	1460	0.24
1,4-cineole	1008	0.22	furfuryl heptanoate	1468	0.11
1,8-cineole	1024	0.18	dimethyl acetal undecanal	1474	0.25
salicylaldehyde	1038	0.15	benzyl acetylacetate	1487	2.45
isobutyl angelate	1044	0.41	*Z*-γ-macrocarpene	1511	0.15
2-acetylpyrrole	1054	0.09	2*E*,4*E*-dodecadienal	1519	0.69
trans-arbusculone	1066	0.58	*Z*-nerolidol	1533	0.28
p-tolualdehyde	1080	0.62	β-calacorene	1563	3.32
o-guaiacol	1087	0.49	hexyl octanoate	1581	1.18
α-fenchocamphorone	1104	0.49	dodecyl acetate	1605	0.54
endo-fenchol	1114	0.19	butyl anthranilate	1616	0.47
α-campholenal	1121	0.14	Benzophenone	1629	0.09
geijerene	1138	0.08	Daucol	1645	0.18
veratrole	1139	0.06	Pogostol	1652	0.28
2-ethyl hexyl acetate	1141	0.07	Ageratochromene	1659	0.15
santolinyl acetate	1172	0.48	Tetradecenol	1670	0.37
furfuryl acetone	1181	0.57	occidentalol acetate	1682	0.65
4*Z*-decenal	1194	0.33	Cyperotundone	1695	0.95
2*E*,4*E*-nonadienal	1208	0.18	4*E*-methoxy cinnamic acid	1700	0.83
2*E*,4*E*-nonadienol	1221	1.29	2*E*,6*Z*-farnesal	1712	0.15
nor-davanone	1231	1.24	methyl tetradecanoate	1721	1.63
o-anisaldehyde	1239	1.65	*E*-coniferyl alcohol	1736	0.09
pregeijerene B	1276	46.89	2*E*,6*E*-farnesol	1748	0.48
o-acetanisole	1291	0.86	Drimenol	1767	4.61
iso-menthyl acetate	1301	1.37	butyl dodecanoate	1788	0.14
2*E*,4*E*-decadienal	1316	1.16	Callitrin	1803	5.51
anisyl formate	1336	0.22	isopropyl tetradecanoate	1822	0.23
4'-methoxy-acetophenone	1349	0.15	Cyclopentadecanolide	1830	0.21
Angustione	1371	2.13	*Z*-ternine	1841	0.37
methyl perillate	1392	0.29	*Z*-lanceol acetate	1857	1.42
methyl cresol acetate	1402	0.31	benzyl salicylate	1867	1.69
cycloseychellene	1407	0.47			

*RI = linear temperature program retention index on DB-5 column.

nor-davanone, 1,4-cineole and 1,8-cineole that was antifungal activity function correlated by cellular membrane damage and leakage inhibiting spore germination, proliferation, and cellular respiration. This study was indicating that the efficiency of *Fusarium* sp. MFLUCC16-1462 possible using as biological control agent in sustainable rice production system.

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