

# Antioxidant and Antifungal Activity of Endophytic Fungi Associated with Agarwood Trees<sup>1</sup>

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## ABSTRACT

Several species of *Aquilaria* and *Gyrinops* are native to Indonesia and well known as agarwood-producing trees with a high economic value. Their bioactive compounds have a wide spectrum of uses, such as in medicine and cosmetics. These genera have undergone extensive search for novel bioactive compounds. The purpose of this study was to isolate, identify, and characterize the endophytic fungi community associated with *Aquilaria malaccensis*, *A. microcarpa*, *Gyrinops versteegii*, and *A. crassna* trees and investigate their bioactive properties as antioxidant agents and antagonists. A total of 50 fungi were successfully isolated from different tissues of the four species of agarwood-producing trees. Two isolates exhibited strong antioxidant activity, namely, *Apodus oryzae* (R2MC3A, IC<sub>50</sub> 60.92 mg/mL) and *Diaporthe* sp. (PIDS1[C], IC<sub>50</sub> 76.65 mg/mL). Two isolates, *Pestalotiopsis theae* (P3BS3[B]) and *Curvularia* sp. (P2CD3A), showed >75% antifungal activity against pathogenic *Fusarium solani*. The results revealed that endophytic fungi associated with the studied agarwood-producing trees had potential antioxidant and antifungal activities for further applications in biotechnology.

**Keywords:** *Aquilaria*, *Gyrinops*, endophytic fungi, antioxidant, antagonists

## 1. INTRODUCTION

*Thymelaeaceae* is the major plant family producing agarwood, also called Gaharu in Indonesian. Agarwood have been used as one of the essential ingredients in fragrance, aromatherapy, pharmaceutical and herbal medicines for centuries. It also hold social, cultural, and economic values in local communities (Beek and Philips, 1999; Barden *et al.*, 2000; Donovan and Puri,

2004). Indonesian agarwood has been known worldwide and retained long historical story. It was first recorded as the main commodity bartered between the empires of China and the kingdoms of Indonesia since the Silk Road era (Turjaman *et al.*, 2016). Wider spectrums of agarwood uses have been known for sedative in oriental medicine, antimicrobial, antitumor and antioxidant activity (Takemoto *et al.*, 2008; Wetwitayaklung *et al.*, 2009; Chen *et al.*, 2011). Agarwood is the resinous

<sup>1</sup> Date Received March 16, 2019, Date Accepted July 15, 2019

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and fragrant heartwood that may not be formed in normal wood tissue but formed in wounded trees. Among them, *Aquilaria* and *Gyrinops* are the most common agarwood-producing genus in Indonesia (Gong and Guo, 2009). Different parts of various plant have been known to have various benefit (Jung et al., 2017. Ham and Kim, 2018; Li et al., 2018), healthy wood of *Aquilaria* and *Gyrinops* have been acknowledge to contain bioactive compound; leaves demonstrated inhibition activity against polymorphonuclear neutrophils (PMNs) respiratory burst stimulated by PMA (Qi et al., 2009), anti-diabetic, anti-HIV, anti-cancer, immuno-modulatory anti-inflammatory and laxative effects (Yoshimi et al., 2001); while fresh steam exhibited cytotoxicity against SGC-7901 and SMMC-7721 cell lines (Wang et al., 2010).

Parallel with the huge loss of agarwood producing-tree in the wild, the losses of endophytic fungi associated with the trees automatically determine the loss of Indonesia biodiversity as well. Agarwood-producing trees are host for many endophytic fungi which produce similar or even higher amount of bioactive substrates compare to their host plant (Stierle et al., 1993; Strobel et al., 1996; Li et al., 1998). Previous studies have recorded that 1) *Fusarium* spp. isolated from leaves, stem and root of *A. sinensis* showed the most potent antimicrobial activity, 2) several endophytes fungi (Gong and Guo, 2009) isolated from *A. crassna* showed capability to produce indole-3-acetic acid (IAA) and siderophores (Nimnoi et al., 2010), 3) *F. verticillioides* SHTTr3 and *Colletotrichum truncatum* SHTTrHc7 isolated from *A. crassna* showed comparable scavenging abilities on DPPH-free radicals (Chi et al., 2016), 4) *Xylaria mali*, *Lasiodiopodia theobromae* and *Phaeoacremonium rubrigenum* isolated from *A. sinensis* exhibited cytotoxicity against 293-T, 293-T, and SKVO3 cells lines (Cui et al., 2011), and 5) fungus AL-2 strain isolated from *A. malaccensis* had antibacterial activities against gram positive and negative bacteria as well as general

toxicity based on brine shrimp lethality (Shoeb et al., 2010). *A. sinensis* have been used as traditional Chinese medicinal, a tropical evergreen tree distributed in Hainan, Guangdong, Guangxi, Yunnan and Taiwan (Chen et al., 2016); *A. crassna* used also as traditional medical plant and widely distributed in Indochina and Thailand (Turjaman and Hidayat, 2017); and so that for *A. malaccensis* which is the most popular species for its agarwood production and distributed from different countries, Bangladesh, Bhutan, Cambodia, India, Indonesia, Iran, Lao PRD, Malaysia, Myanmar, Philippines, Singapore, Thailand, Vietnam and Papua New Guinea (Oldfield et al., 1998; Premalatha and Kalra, 2013).

The genus *Aquilaria* has 27 species spread worldwide, which 6 are found in Indonesia (Soehartono and Newton, 2000; Saikia and Khan, 2013). The genus *Gyrinops* is distributed in at least 12 countries, and 7 species were found naturally in Indonesia (Barden et al., 2000). Several studies have been previously conducted to those species, particularly on exploration and investigation of their phytochemical constituents and their respective endophytic fungi bioactivities. There was vast variations of endophytic fungal communities related to living conditions, host plant species, as well as host tissue types (Lamit et al., 2014); indicating that further investigations are important. For this reason, this study aimed to isolate the understudied endophytic fungi associated with three Indonesia-native species of agarwood (*A. malaccensis* and *A. microcarpa*, *G. versteegii*) and one exotic species (*A. crassna*), as well as their antioxidant and antifungal activities.

## 2. MATERIALS and METHODS

### 2.1. Chemicals

Alcohol, NaClO 4%, *Dextrose*, KOH 10%, HCl, Glycerol 20%, acetic acid anhydrite, magnesium (Mg),

H<sub>2</sub>SO<sub>4</sub>, Folin-Ciocalteu (FC), ethyl acetate and methanol were purchased from Merck chemical (Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich chemical (USA) and other chemicals were provided by Himedia chemicals (India).

## 2.2. Samples and cultures medium for isolation

Different parts of plant materials (healthy segment; bark, leaves, shoot and root) from *Aquilaria* and *Gyrinops* trees originated from West Java and Bangka were collected (diameter breast high /dbh > 15 cm, either grown naturally or planted). The potato dextrose agar (PDA), which consist of potato extract 20%, dextrose 2%, and agar 2% was used for isolation. Pure fungal strains were sub-cultured and maintained on PDA prior to use. Malt Yeast Glucose Agar (MYGA, containing yeast extract 0.4%, malt extract 1%, sucrose 0.4%, and agar 2%), and Malt Yeast Glucose Pepton Agar (MYGPA, which consist of yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, sucrose 1.05%, and agar 2%) were also used to obtain optimum fungal growth (Atlas, 2004).

## 2.3. Endophytic fungal isolation

Fungal strains were isolated from bark, leaves, shoot, and root of *A. malaccensis*, *A. microcarpa*, *A. crasna* and *G. verstigi* trees. The tree parts were collected at least from three different trees. In order to minimize the growth of epiphytic fungi during internal tissue sampling, tree parts were surface-sterilized by washing, followed by soaking in alcohol 70% for 2 min, and lastly in sodium hypochlorite for 2 min (4% Cl active). The outer tissues were removed, and the inner tissues of 0.5 x 0.5 cm size were carefully dissected and placed in PDA media plates. Chloramphenicol (100 mg/L) was added to media to suppress bacterial growth. The plates were then incubated at room temperature for up to 14 days, during which the development of different colonies

was periodically observed and verified. Each colonies of different fungi appeared from tissue fragment were cut out by dissecting microscope and purified on PDA plates. All pure cultures obtained were used for further investigation.

To select the most suitable medium for each isolate, the pure cultures were tested on PDA, MYGA and MYGPA media. A disk (5 mm, diameter) of actively growing fungal colony on agar media was obtained with a cork-borer and was sub-cultured into petri dish containing PDA, MYGA and MYGPA media. The plate was incubated for 7 d, and the fungal growth was observed daily. All the experiments were conducted in triplicate.

## 2.4. Screening of endophytic fungi against *F. solani* in dual culture

*F. solani* was obtained from Laboratory of Forest Microbiology (INTROF-CC 00509, Forest Research and Development Centre, Bogor, Indonesia) All pure strains of fungal endophytes were screened against *F. solani* in dual cultures on PDA. An agar disk (5 mm diameter) of endophytic and *F. solani* was co-inoculated 4 cm apart on PDA in petri dish. The plates were incubated at room temperature up to 7 days. All experiments were carried out in triplicates. Growth inhibition percentage was determined by the following formula (Dennis and Webster, 1971).

$$\text{Percentage of inhibition} = [(J1-J2)/J1] \times 100\%$$

Where J1 is radial covered by pathogen toward petri dish wall (outward), and J2 is radial covered by pathogen (*F. solani*) toward endophytic fungi.

## 2.5. Screening of fungal endophytes for antioxidant activity

### 2.5.1. Preparation of fungal extraction

Each of endophytic fungal isolates was grown in 250

mL Erlenmeyer flasks containing 50 ml of selected liquid medium (previously described) and incubated for 14 days in stationary condition. After incubation, mycelia biomass was harvested by filtering through Whatman No. 1 filter paper. The filter papers containing mycelia mat were oven dried until constant weight was reached, then extracted by ethyl acetate. The filtrate was also extracted by ethyl acetate using funnel separation. Each organic phase was mixed, and the solvent was then removed under reduced pressure using rotary vacuum evaporator to obtain a concentrate. The yield of ethyl acetate soluble was determined by calculation with dried weight of fungal mycelium (% w/w) and volume of culture liquid medium (% w/v).

### 2.5.2. Antioxidant assay

Antioxidant activity of each extract was determined according to published methods with slight modifications (Kamonwannasit *et al.*, 2013; Marson Ascêncio *et al.*, 2014; Sadeghi *et al.*, 2015). In a series of test tubes, extract was diluted with ethyl acetate to obtain in the range of concentration into which 0.25 mL of a DPPH (1 M) and added methanol to obtain 2.5 mL of final reaction volume. After 30 min incubation at room temperature, the absorbance was monitored at 517 nm. The ethyl acetate solution without extracts was selected as control. All experiments were conducted in triplicated test. The inhibitory activity was calculated from  $[(A_0 - A_1)/A_0] \times 100$ , where  $A_0$  is the absorbance of the control, and  $A_1$  is the absorbance of the extract or standard. The antioxidant activity was expressed as  $IC_{50}$ , in which the amount of sample necessary to reduce the initial DPPH concentration by half.

### 2.5.3. Phytochemical assay

Phytochemical assay was carried out according to Tapwal *et al.* (2016) with slight modification, including detection of some important constituents (phenolic, flavonoid, tannin, steroid, terpenoid, and alkaloid). All ethyl acetate soluble obtained from extraction (pre-

viously described) with concentration of 1000  $\mu\text{g/L}$  was subjected for phytochemical assay. In general, phenolic and tannin were reacted by ferric chloride test, flavonoid was determined by addition of magnesium and hydrochloric acid, steroid was monitored by addition of acetate anhydrate and sulfuric acid, terpenoid was determined by addition of sulfuric acid, and alkaloid was monitored by using Mayer's and Wagner's reagents.

## 2.6. Identification of fungi

Fungal identification was done with molecular methods, focusing on fungi with antioxidant and antifungal properties. DNA was obtained by extraction of mycelia cultured in 7 d grown on potato dextrose broth (PDB) using DNA Wizard Kit (Promega, USA) according to the published method (Kumar and Shukla, 2005). The internal transcribed spacer ITS region (Jellison and Jasalavich, 2000) was amplified by polymerase chain reaction (PCR) using ITS1 (5'-CTTGGTCATTTAG AGGAAGTAA-3') and ITS4-B (5'-CAGGAGACTT GTACAGGTCCAG-3'). The PCR was performed in 20  $\mu\text{l}$  of solution containing 10 ng of genomic DNA, 5 pmol each of the forward and reverse primers, and 10  $\mu\text{l}$  of Go Taq® Hot Start Colourless Master Mix (Promega, Wisconsin USA) according to the manufacturer's instructions. Prior to sequencing, the PCR products were purified using rAPid Alkaline Phosphatase (Roche, Germany) and exo-nuclease I (New England Biolabs, Massachusetts, USA). The purified PCR products were Sanger sequenced (First Base Sequencing Service, Singapore). Homology searches were conducted by using the BLASTn program in the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/>).

## 2.7. Statistical analysis

All results were presented as the mean  $\pm$  the standard deviation. When necessary, results were also analysed

using SPSS (version 15) for Windows.

### 3. RESULTS and DISCUSSION

#### 3.1. Endophytic fungi isolation

Total of 50 endophytic fungal isolate was successfully purified from 232 different part of fresh and healthy plant material belonging to *A. malaccensis*, *A. microcarpa*, *A. crassna* and *G. Versteegii* collected from West Java and Bangka Belitung Provinces. There were 24, 16, 7 and 3 pure endophytic fungal isolates from bark, leaves, shoot, and root, respectively. All fungal isolates were able to grow in all three media, with most of them showed highest colony growth in PDA (80%), MYGA (14%) and MYGPA (6%) (data not shown here). Hence, PDA and its equivalent liquid medium, potato dextrose broth (PDB), were selected for further investigation on antioxidant and antifungal properties. Endophytic microorganisms, mostly fungi in general, are localized internally in their host plants and become an important issue in the past few decades (Faeth, 2002; Staniek *et al.*, 2008). Endophytic fungi thrive asymptotically in the tissues of plants including stems, leaves, barks and/or roots. They may help clarify the role and biological change in stressful environments. Pure endophytic fungal isolates from *G. versteegii* were most abundant (21 strains) among other host plant species in this study; *A. crassna* (14 strain), *A. microcarpa* (7 strains) and *A. malaccensis* (3 strains). The isolation of endophytic fungi by traditional method in this study have revealed a new finding, particularly originated from *G. versteegii* and *A. microcarpa*, which has never been conducted before and could serve as a reference for the next studies. Whereas the diversity of endophytic fungi and their various potential bioactive metabolites from *A. crassna* and *A. malaccensis* in this study has contributed to wider knowledge of the potential use of endophytic fungi for agarwood-producing trees as

has been previously reports (Nimnoi *et al.*, 2010; Shoeb *et al.*, 2010; Chi *et al.*, 2016).

#### 3.2. Antioxidant activity

In this study, 50 endophytic fungal isolates were evaluated for antioxidant activity. The results revealed that among 50 isolates, six had ability to reduce the absorbance of DDPH free radical with the range was 36% - 82% (Table 1); P1C1S3 ( $39.87 \pm 2.61$ ), P2CD3B ( $36.25 \pm 0.01$ ), P1DS1[C] ( $65.25 \pm 0.73$ ), P3DS1[A] ( $46.5 \pm 2.33$ ), P1B1C ( $40.67 \pm 0.73$ ), and R2MC3A ( $82.08 \pm 5.99$ ). Further analysis (Table 1) indicated that the ethyl acetate extracts from two isolates, R2MC3A and P1DS1[C] had  $IC_{50}$  of 60.92  $\mu\text{g/mL}$  and 76.65  $\mu\text{g/mL}$ , fall into the range of strong category (50-100  $\mu\text{g/mL}$ ). Whereas other isolates those had  $IC_{50}$  fall under the range of medium category (100-150  $\mu\text{g/mL}$ ). The ethyl acetate extract of R2MC3A resulted in higher yield, 0.06% (w/v) or 11.97% w/w of dried mycelia, than of P1DS1[C] (Table 2). These results indicated that the highest potential antioxidant was generated from endophytes isolated from fresh leaves (P1DS1[C]) and roots (R2MC3A) of *G. versteegii* and *A. microcarpa* trees.

Molecular identification clearly identified *Diaporthe* sp. (P1DS1[C]) and *Apodus oryzae*. (R2MC3A), both have  $IC_{50}$  less than 80  $\mu\text{g/mL}$  (Table 3). Interestingly, *Diaporthe* sp. has been known as a genus of plant pathogenic, saprobic and endophytic fungi (Gomes *et al.*, 2013); while *Apodus oryzae* (R2MC3A) has been known as aminor endophytic and rhizospheric fungi associated with several plants (Cai *et al.*, 2006; Zhou *et al.*, 2015). In contrast, *Apodus oryzae* isolate (R2MC3A) showed greater scavenging activity ( $IC_{50}$  60.92  $\mu\text{g/mL}$ ) than *Diaporthe* sp. (P1DS1[C]) isolate. This study is the first report to discover that *Apodus oryzae* (R2MC3A) and *Diaporthe* sp. played as endophytic fungi which resided in the leaves tissue of

**Table 1.** Antioxidant and antifungal activity of thirteen potential endophytic fungi

Explants	Host Plants	Isolates	Antioxidants			Antifungal
			Inhibitory Activity (%)*	IC <sub>50</sub> (µg/mL)	Categories**	(%)
Bark	G. versteegii	1 P3C2S3 [B]	18.37 ± 7.058	>200	1	71.42 ± 0.82
		2 P1C1S3 [B]	39.87 ± 2.611	125.41	3	49.39 ± 5.57
	A. crassna	1 P1CD2B	11.59 ± 1.753	>200	1	72.00 ± 0.00
		2 P2CD3A	7.42 ± 1.244	>200	1	77.00 ± 4.24
		3 P2CD3B	36.25 ± 0.004	137.93	3	64.00 ± 5.66
		4 P2CK1B	10.50 ± 0.121	>200	1	73.67 ± 13.67
Leaves	G. versteegii	1 P1DS1[C]	65.24 ± 0.728	76.65	4	59.21 ± 11.57
		2 P3DS1[A]	46.50 ± 2.333	107.53	3	61.11 ± 7.85
	A. crassna	1 P1D2A	6.44 ± 0.155	>200	1	72.54 ± 0.76
	A. microcarpa	1 LMC3D11	6.58 ± 0.280	>200	1	70.00 ± 2.31
shoot	G. versteegii	1 P3BS3 [B]	14.44 ± 3.611	>200	1	78.46 ± 7.84
	A. crassna	1 P1B1C	40.60 ± 0.728	123.15	3	51.65 ± 7.77
Root	A. microcarpa	1 R2MC3A	82.08 ± 5.990	60.92	4	9.17 ± 1.44

Note: \* concentration of fungal crude extract (100 mg/L);

\*\* 1 = very weak, 2 = weak, 3 = medium, 4 = strong, 5 = very strong

*G. versteegii* and the roots of *A. microcarpa*. Previous studies reported that only *Fusarium*, *Colletotrichum*, *Xylaria*, *Lasiodiplodia*, *Phaeoacremonium*, have been isolated from *A. sinensis*, *A. crassna* and *A. malaccensis* (Gong and Guo, 2009; Nimnoi *et al.*, 2010; Shoeb *et al.*, 2010; Cui *et al.*, 2011; Chi *et al.*, 2016). *Diaporthe* sp. has been previously described as endophyte and produce bioactive secondary metabolites which are antioxidant potential. However, antioxidant activity between isolates varies greatly. For example, *Diaporthe* sp isolated from *Costus spiralis* have shown antioxidant activity (IC<sub>50</sub>) between 500-1750 µg/mL (Marson Ascêncio *et al.*, 2014), whereas IC<sub>50</sub> of 76.65 µg/mL were obtained from *Diaporthe* sp (P1DS1[C]) isolated from leaves tissue of *G. versteegii*. *Diaporthe* sp. was reported to produce bioactive related to phenolic compounds, such as benzene acetaldehyde, benzyl benzoate, salicylaldehyde, benzoin and benzyl cinnamate (Tanapichatsakul *et al.*, 2018).

Other interesting finding is the antioxidant capacity of *Apodus* sp. *Apodus* sp. was previously reported to be found in *Hedyotis diffusa* and *Pseudotsuga menziesii* (Zhou *et al.*, 2015; Daniels, 2017). However, the information on its antioxidant activity was not yet investigated. The antioxidant activity of *Apodus oryzae* (R2MC3A), among other isolates in this study, was the highest (60.92 µg/mL) and confirmed to contain a phenolic compound (Table 2); achieving the total phenolic content (TPC) 96.10 mg gallic acid equivalent per g sample (data not shown here). The detection of TPC content clearly supported other findings on antioxidant capacity closely related with TPC (Sadeghi *et al.*, 2015; Jeong *et al.*, 2017; Hidayat *et al.*, 2018; Kim *et al.*, 2018). Although, the specific phenolic compound was not yet identified, the phenolic content itself is important due to its unique structure for redox property which acts as high reducing agents, hydrogen donors, and singlet oxygen quenchers which may lead

**Table 2.** Biomass, yield and phytochemical analysis of crude extract ethyl acetate soluble of 13 isolates endophytic fungi after fermentation at 14 days

Explants	Host Plants	Isolates	Mycelium Weight (mg/L)	Yield of Ethyl Acetate Soluble Extracts		Phytochemical Properties					
				% (w/v)*	% (w/w)**	Alkaloid		Flavonoid*	Fenol*	Terpenoid	Steroid
						Mayer	Wagner				
Bark	<i>G. versteegii</i>	1 P3C2S3 [B]	6,354	0.02	3.02	-	-	+	+	+	-
		2 P1C1S3 [B]	59,813	0.07	1.14	-	-	+	+	-	+
	<i>A. crassna</i>	1 P1CD2B	9,957	0.05	4.86	-	-	-	+	+	-
		2 P2CD3A	9,083	0.04	4.36	-	-	+	-	+	+
	<i>A. crassna</i>	3 P2CD3B	5,850	0.03	4.99	-	-	-	+	+	-
		4 P2CK1B	5,570	0.03	6.13	-	-	+	+	+	-
Leaves	<i>G. versteegii</i>	1 P1DS1[C]	7,461	0.03	4.66	-	-	+	+	+	-
		2 P3DS1[A]	6,082	0.06	10.06	-	-	+	+	+	-
	<i>A. crassna</i>	1 P1D2A	4,315	0.06	13.52	-	-	+	-	+	-
	<i>A. microcarpa</i>	1 LMC3D11	10,395	0.02	2.20	-	-	-	+	-	+
Shoot	<i>G. versteegii</i>	1 P3BS3 [B]	6,936	0.03	3.81	-	-	+	-	+	-
	<i>A. crassna</i>	1 P1B1C	3,069	0.04	12.49	-	-	-	+	+	-
Root	<i>A. microcarpa</i>	1 R2MC3A	4,691	0.06	11.97	-	-	-	+	-	+

Noted: \* calculated with volume of liquid medium, \*\* calculated with mycelium weight, + = indicates presence, - = indicates absence

to antioxidant activity (Khoddami *et al.*, 2013; Kim *et al.*, 2017).

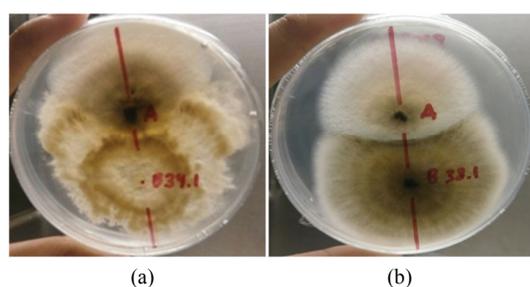
### 3.3. Antifungal against *F. solani* activity

Antifungal activity tested by means of dual culture/confrontation against *F. solani*. *Fusarium* was recorded as a significant plant pathogen and an inducer of agarwood formation (Sitepu *et al.*, 2011; Turjaman *et al.*, 2016). This pathogenic fungi was commonly used to induce agarwood formation and one of the most effective agent for agarwood formation in Indonesia (Turjaman *et al.*, 2016). The effectiveness of agarwood formation was affected by many factors such as host tree, type of fungal pathogen, and environment (Santoso, 2013), as well as the presence of endophytic fungi inside the host tree (Nimnoi *et al.*, 2011; Zhang *et al.*, 2014). In this study, 50 endophytic fungi showed variation

in the capability to inhibit the growth of *F. solani* (9-78%). Seven isolates inhibited the growth of *F. solani* over than 70%, (Table 1), those were P2CK1B (73%), P1D2A (72%), P1CD2B (72%), P2CD3A (77%), P3BS3[B] (78%), P3C2S3[B] (71%), and LMC3D11 (70%). Most above-ground tree isolates had strong antifungal activity against *F. solani*, while R2MC3A, which was originated from the root, had a weak antifungal activity. It revealed that R2MC3A was not compatible to work against *F. solani*, which produced symptoms on the infected root (Sitepu *et al.*, 2011; Turjaman *et al.*, 2016). More detailed observation on tissue origin and host plant species revealed that the strongest inhibitions (> 75%) were originated from shoot (P3BS3[B]) and bark (P2CD3A) of *G. versteegii* and *A. crassna* trees. According to molecular identification (Table 3), isolate P3BS3[B] and P2CD3A were clearly identified as *P. theae* and *Curvularia* sp.

**Table 3.** Identification of 13 potential endophytic fungi based on internal transcribed spacer (ITS) using BLAST analysis

Explants	Host plants	Isolates	Similarity (%)	Species	Accession number	Division, class, family
Bark	<i>G. versteegii</i>	1 P3C2S3 [B]	-	-	-	-
		2 P1C1S3 [B]	99	<i>Daldinia eschscholtzii</i>	KT936498	Ascomycota, Ascomycetes, Xylariaceae
	<i>A. crassna</i>	1 P1CD2B	-	-	-	-
		2 P2CD3A	100	<i>Curvularia</i> sp.	KP377591	Ascomycota, Euascomycetes, Pleosporaceae
Leaves	<i>G. versteegii</i>	1 P1DS1[C]	100	<i>Diaporthe</i> sp	EF423549	Ascomycota, Sordariomycetes, Diaporthaceae
		2 P3DS1[A]	100	<i>Colletotrichum magnum</i>	KC815123	Ascomycota, Sordariomycetes, Glomerellaceae
	<i>A. crassna</i>	1 P1D2A	99	<i>Phyllosticta capitalensis</i>	KP900294	Ascomycota, Dothideomycetes, Botryosphaeriaceae
	<i>A. microcarpa</i>	1 LMC3D11	99	<i>Diaporthe</i> sp	EF423549	Ascomycota, Sordariomycetes, Diaporthaceae
Shoot	<i>G. versteegii</i>	1 P3BS3 [B]	100	<i>Pestalotiopsis theae</i>	EU833970	Ascomycota, Sordariomycetes, Pestalotiopsidaceae
	<i>A. crassna</i>	1 P1B1C	-	-	-	-
Root	<i>A. microcarpa</i>	1 R2MC3A	100	<i>Apodus oryzae</i>	KU059868	Ascomycota, Sordariomycetes, Sporacaceae



**Fig. 1.** Dual culture assay between potential endophytic against the pathogen of *F. solani* after 6 days incubation. A. *P. theae* P3BS3[B], B. *Curvularia* sp. (P2CD3A).

The interaction between two potential endophytic and pathogenic fungi showed in Fig. 1. It was categorized

as type E interaction (Wheeler and Hocking, 1993), where the mycelia of *P. theae* and *Curvularia* sp. continued to grow, covered and reduced pathogen's colony. In the *Curvularia* sp - *F. solani* interaction, green black color appeared at the border of the two colonies. Yellow brown was appeared circling the mycelium of *P. theae* interacted with *F. solani*. This phenomenon showed that both colonies competed to access nutrients, secreted certain enzymes, and produced bioactive compounds to cease the opponent's growth by changing of fungal cell membrane (Hamzah *et al.*, 2018; Kim *et al.*, 2016). As an indicator of bioactive compound production, the phytochemical assay from ethyl acetate extract of fungal culture of *P. theae* and *Curvularia*

sp. assayed and showed a positive result to flavonoid, terpenoid and/or steroid constituent (Table 2). Compounds constituents from endophytes of Genus *Pestalotiopsis* and *Curvularia* have been previously reported (Tan and Zou, 2001; Kaul *et al.*, 2012; Mondol *et al.*, 2017; Alurappa and Chowdappa, 2018; Chutulo and Chalannavar, 2018; Deshmukh *et al.*, 2018). *Pestalotiopsis* sp. with the huge diversity of endophytic fungi has appeared as potential source of bioactive natural compounds. They have been reported to produce various compounds such as alkaloids, terpenoids, flavonoids, and phenols, which has been reported to have antimicrobial, antifungal, antiviral antineoplastic, and antioxidant activities (Deshmukh *et al.*, 2017). The terpenoid compounds isolated from the endophyte *Pestalotiopsis* sp. was the most abundant compound among other constituents, which are moderate to high antifungal property, such as 10-Deacetylbaaccatin III, Pestalofone C, Pestalofone E, (3R,4R,6R,7S)-7-hydroxyl-3,7-dimethyl-oxabicyclo[3.3.1]nonan-2-one, and (3R,4R)-3-(7-methylcyclohexenyl)-propanoic acid (Deshmukh *et al.* 2017, 2018). *Curvularia* sp. isolated from *Rauwolfia tetraphylla* L. was reported to contain alkaloid and terpenoid, and had antifungal activity (Alurappa and Chowdappa, 2018). Pyrenolide A produced from *Curvularia* sp., strain M12, was isolated from *Murraya koenigii* and showed strong activity against *Phytophthora capsici* (Mondol *et al.*, 2017). Studies on agarwood production in genera *Aquilaria* and *Gyrinops* reported that only 7-10% of tree contains agarwood, which sesquiterpenes and 2-(2-phenylethyl)-4H-chromen-4-one was the major chemical constituent (Naef, 2011; Chen *et al.*, 2012). Although terpenoids have been found in the host plant (agarwood) as well as on the potential endophytic fungi in this study, the relationship between them are still unclear; whether it is mutualistic, symbiotic, antagonistic, or slightly pathogenic.

## 4. CONCLUSION

Fitty endophytic fungi were successfully isolated from different parts of *A. malaccensis*, *A. microcarpa*, *A. crassna* and *G. verstegii* trees grown in West Java and Bangka Belitung Provinces, Indonesia. Among them, *Diaporthe* sp. (P1DS1[C]) and *A. oryzae*. (R2MC3A) originated from fresh leave *G. verstegii* and root of *A. microcarpa* produced phenolic chemical groups which have antioxidant activities (IC<sub>50</sub>) less than 80 µg/mL. Two other isolates, *P. theae* (P3BS3[B]) and *Curvularia* sp (P2CD3A) originated from fresh shoot of *G. verstegii* and bark of *A. crassna* showed antifungal activity against the phytopathogenic fungi *F. solani*, potentially by producing terpenoid chemical groups constituent. New finding of the occurrence of endophytic fungi (*Diaporthe* sp., *A. oryzae*, *P. theae*, *Curvularia* sp.) may be used as the bioresources for antioxidant and also as antifungal agents for wider biotechnology applications in the future.

## ACKNOWLEDGMENT

This work was partly supported by Ministry of Environmental and Forestry, Republic of Indonesia, FY 2016. We thanks to our colleagues for helping and supporting to our research work and critical comments in writing of manuscript. Asep Hidayat and Maman Turjaman conceived and designed the experiments, collected samples, performed the experiments, analysed and interpreted the data, contributed reagents, materials, analysis tools, wrote and edited the manuscripts. Sarah Asih Faulina helped with fungal identification, wrote and edited the manuscripts. Fadel Ridwan, Aryanto, Najmulah collected samples and performed experiments. Tun Tedja Irwanti wrote and edited the manuscripts. All authors reviewed and approval final manuscripts.

## REFERENCES

- Alurappa, R., Chowdappa, S. 2018. Antimicrobial activity and phytochemical analysis of endophytic fungal extracts isolated from ethno-pharmaceutical plant *Rauwolfia tetraphylla* L. Ramesha. *Journal of Pure and Applied Microbiology* 12(1): 317-332.
- Atlas, R.M. 2004. *Handbook of microbiological media*. CRC Press, Florida, USA.
- Barden, A., Anak, N.A., Mulliken, T., Song, M. 2000. Heart of the matter: agarwood use and trade and CITES implementation for *Aquilaria malaccensis*. Cambridge : TRAFFIC International.
- Beek, H.H.v., Philips, D. 1999. *Agarwood: trade and CITES implementation in Southeast Asia*.
- Cai, L., Jeewon, R., Hyde, K.D. 2006. Phylogenetic investigations of Sordariaceae based on multiple gene sequences and morphology. *Mycological Research* 110(2): 137-150.
- Chen, G., Liu, C., Sun, W. 2016. Pollination and seed dispersal of *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae): An economic plant species with extremely small populations in China. *Plant Diversity* 38(5): 227-232.
- Chen, H., Yang, Y., Xue, J., Wei, J., Zhang, Z., Chen, H. 2011. Comparison of compositions and antimicrobial activities of essential oils from chemically stimulated agarwood, wild agarwood and healthy *Aquilaria sinensis* (Lour.) Gilg trees. *Molecules* 16(6): 4884-4896.
- Chen, H.Q., Wei, J.H., Yang, J.S., Zhang, Z., Yang, Y., Gao, Z.H., Sui, C., Gong, B. 2012. Chemical constituents of agarwood originating from the endemic genus *Aquilaria* plants. *Chemical & Biodiversity* 9(2): 236-250.
- Chi, H.K., Cuong, L.H., Hang, T.T.N., Luyen, N.D., Ha, T.T.H., Huong, L.M. 2016. Biological characterization of fungal endophytes isolated from agarwood tree *Aquilaria crassna* Pierre Ex Lecomte. *Vietnam Journal of Biotechnology* 14(1): 149-156.
- Chutulo, E.C., Chalannavar, R.K. 2018. Endophytic mycoflora and their bioactive compounds from *Azadirachta Indica* : A comprehensive review. *Journal of Fungi* 4(2): 1-12.
- Cui, J., Guo, S., Xiao, P. 2011. Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. *Journal of Zhejiang University Science B* 12(5): 385-392.
- Daniels, H.A. 2017. Phylogenetic identification of pathogenic and endophytic fungal populations in west Coast Douglas-fir (*Pseudotsuga menziesii*) Foliage. Oregon State University.
- Dennis, C., Webster, J. 1971. Antagonistic properties of species-groups of *Trichoderma*: III. Hyphal interaction. *Transaction of the British Mycological Society* 57(3): 363-369.
- Deshmukh, S., Gupta, M., Prakash, V., Saxena, S. 2018. Endophytic Fungi: A source of potential antifungal compounds. *Journal of Fungi* 4(3): 77.
- Deshmukh, S.K., Prakash, V., Ranjan, N. 2017. Recent advances in the discovery of bioactive metabolites from *Pestalotiopsis*. *Phytochemistry Reviews* 16(5): 883-920.
- Donovan, D.G., Puri, R.K. 2004. Learning from traditional knowledge of non-timber forest products: Penan Benalui and the Autecology of *Aquilaria* in Indonesian Borneo. *Ecology and Society* 9(3): 3.
- Faeth, S.H. 2002. Are endophytic fungi defensive plant mutualists? *Oikos* 98: 25-36.
- Gomes, R.R., Glienke, C., Videira, S.I.R., Lombard, L., Groenewald, J.Z., Crous, P.W. 2013. *Diaporthe*: A genus of endophytic, saprobic and plant pathogenic fungi. *Persoonia-Molecular Phylogeny and Evolution of Fungi* 31: 1-41.
- Gong, L.J., Guo, S.X. 2009. Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. *African Journal of*

- Biotechnology 8(5): 731-736.
- Ham, Y., Kim, T-J. 2018. Plant extracts inhibiting biofilm formation by *Streptococcus mutans* without antibiotic Activity. Journal of the Korean Wood Science and Technology 46(6): 692-702.
- Hamzah, T.N.T., Lee, S.Y., Hidayat, A., Terhem, R., Faridah-hanum, I., Mohamed, R. 2018 Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *Rhizophora mucronata*, and identification of potential antagonists against the soil-borne fungus, *Fusarium solani*. Frontiers in Microbiology 9: 1707.
- Hidayat, A., Iswanto, A.P., Susilowati, A., Rachmat, H.H. 2018. Radical scavenging activity of kemenyan resin produced by an Indonesian native plant, *Syrax sumatrana*. Journal of the Korean Wood Science and Technology 46(4): 346-354.
- Jellison, J., Jasalavich, C. 2000. A review of selected methods for the detection of degradative fungi. International Biodeterioration & Biodegradation 46(3): 241-244.
- Jeong, M-J., Yang, J., Choi, W-S., Kim, J-W., Kim, S-J., Park, M-J. 2017. Chemical compositions and antioxidant activities of essential oil extracted from *Neolitsea aciculata*(Blume) Koidz leaves. Journal of the Korean Wood Science and Technology 45(1): 96-106.
- Jung, J-Y., Yang, J-K., Lee, W-H. 2017. Antioxidant and safety test of natural extract of *Quercus mongolica*. Journal of the Korean Wood Science and Technology 45(1): 116-125.
- Kamonwannasit, S., Nantapong, N., Kumkrai, P., Luecha, P., Kupittayanan, S., Chudapongse, N. 2013. Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. Annals of Clinical Microbiology and Antimicrobials 12(20): 1-7.
- Kaul, S., Gupta, S., Ahmed, M., Dhar, M.K. 2012. Endophytic fungi from medicinal plants: A treasure hunt for bioactive metabolites. Phytochemistry Reviewa 11(4): 487-505.
- Kim, J-W., Um, M., Lee, J-W. 2018. Antioxidant activities of hot water extracts from different parts of *Rugosa rose* (*Rosa rugosa* Thunb.). Journal of the Korean Wood Science and Technology 46(1): 38-47.
- Kim, S-H., Lee, S-Y., Cho, S-M., Hong, C-Y., Park, S-Y., Park, M-J., Choi, I-G. 2017. Antioxidant activities of *Cryptomeria japonica* leaves extracts by extraction Methods. Journal of the Korean Wood Science and Technology 45(5): 495-510.
- Kim, S-H., Lee, S-Y., Cho, S-M., Hong, C-Y., Park, M-J., Choi, I-G, 2016. Evaluation on anti-fungal activity and synergy effects of essential oil and their constituents from *Abies holophylla*. Journal of the Korean Wood Science and Technology 44(1): 113-123.
- Khoddami, A., Wilkes, M.A., Roberts, T.H. 2013. Techniques for analysis of plant phenolic compounds. Molecules 18(2): 2328-2375.
- Kumar, M., Shukla, P.K. 2005. Use of PCR targeting of internal transcribed spacer regions and single-stranded conformation polymorphism analysis of sequence variation in different regions of rRNA genes in fungi for rapid diagnosis of mycotic keratitis. Journal of Clinical Microbiology 43(2): 662-668.
- Lamit, L.J., Lau, M.K., Sthultz, C.M., Wooley, S.C., Whitham, T.G., Gehring, C.A. 2014. Tree genotype and genetically based growth traits structure twig endophyte communities. American Journal of Botany 101 (3): 467-478.
- Li, J.Y., Sidhu, R.S., Ford, E.J., Long, D.M., Hess, W.M., Strobel, G.A. 1998. The induction of taxol production in the endophytic fungus - *Periconia* sp from *Torreya grandifolia*. Journal of Industrial Microbiology and Biotechnology 20(5): 259-264.
- Li, Z-J., Chen, S., Yang, X-H., Wang, R., Min, H-J.,

- Wu, L., Si, C-L., Bae, Y-S. 2018. Secondary metabolites with anti-complementary activity from the stem barks of *Juglans mandshurica* Maxim. Journal of the Korean Wood Science and Technology 46(2): 118-124.
- Ascêncio, P.G.M., Ascêncio, S.D., Aguiar, A.A., Fiorini, A., Pimenta, R.S. 2014. Chemical assessment and antimicrobial and antioxidant activities of endophytic fungi extracts isolated from *Costus spiralis* (Jacq.) Roscoe (Costaceae). Evidence-based Complementary and Alternative Medicine 14(3): 1-10.
- Mondol, M.A.M., Farhouse, J., Islam, M.T., Ffler, A.S., Laatsch, H. 2017. Metabolites from the endophytic fungus *Curvularia* sp. M12 act as motility inhibitors against *Phytophthora capsici* Zoospores. Journal of Natural Products 80(2): 347-355.
- Naef, R. 2011. The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: A review. Flavour and Fragrance Journal 26(2): 73-87.
- Nimnoi, P., Pongsilp, N., Lumyong, S. 2010. Endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lec and screening of plant growth promoters production. World Journal of Microbiology and Biotechnology 26(2): 193-203.
- Nimnoi, P., Pongsilp, N., Lumyong, S. 2011. Actinobacterial community and diversity in rhizosphere soils of *Aquilaria crassna* Pierre ex Lec assessed by RT-PCR and PCR-DGGE. Biochemical Systematic and Ecology 39(4-6): 509-519.
- Oldfield, S., Lusty, C., MacKinven, A. 1998. The world list of threatened trees. World Conservation Press, Cambridge, UK.
- Premalatha, K., Kalra, A. 2013. Molecular phylogenetic identification of endophytic fungi isolated from resinous and healthy wood of *Aquilaria malaccensis*, a red listed and highly exploited medicinal tree. Fungal Ecology 6(3): 205-211.
- Qi, J., Lu, J.J., Liu, J.H., Yu, B.Y. 2009. Flavonoid and a rare benzophenone glycoside from the leaves of *Aquilaria sinensis*. Chemical & Pharmaceutical Bulletin (Tokyo) 57(2): 134-137.
- Sadeghi, Z., Valizadeh, J., Azyzian, S.O., Akaberi, M. 2015. Antioxidant activity and total phenolic content of *Boerhavia elegans* (choisy) grown in Baluchestan, Iran. Avicenna Journal of Phytomedicine 5(1): 1-9.
- Saikia, P., Khan, M.L. 2013. Population structure and regeneration status of *Aquilaria malaccensis* Lam. in homegardens of Upper Assam, northeast India. Tropical Ecology 54(1): 1-13.
- Santoso, E. (2013) Agarwood formation by fungal bioinduction. In: Susmianto A, Turjaman M, Setio P (eds) Track record: agarwood inoculation technology by FORDA. FORDA Press, Bogor, Indonesia, p. 249.
- Shoeb, M., Begum, S., Nahar, N. 2010. Study of an endophytic fungus from *Aquilaria malaccensis* Lamk. Bangladesh Journal of Pharmacology 5(1): 21-24.
- Sitapu, I.R., Santoso, E., Siran, S.A., Turjaman, M. 2011. Fragrant wood gaharu: when the wild can no longer provide. ITTO and FORDA, Bogor, Indonesia.
- Soehartono, T., Newton, A.C. 2000. Conservation and sustainable use of tropical trees in the genus *Aquilaria* I. Status and distribution in Indonesia. Biological Conservation 96(1): 83-94.
- Staniek, A., Woerdenbag, H.J., Kayser, O. 2008. Endophytes: Exploiting biodiversity for the improvement of natural product-based drug discovery. Journal of Plant Interaction 3(2): 75-93.
- Stierle, A., Strobel, G., Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260 (5105): 214-216.
- Strobel, G., Hess, W., Ford, E., Sidhu, R.S., Yang, X.

1996. Taxol from fungal endophytes and the issue of biodiversity. *Journal of Industrial Microbiology* 17(5-6): 417-423.
- Takemoto, H., Ito, M., Shiraki, T., Yagura, T., Honda, G. 2008. Sedative effects of vapor inhalation of agarwood oil and spikenard extract and identification of their active components. *Journal of Natural Medicines* 62(1): 41-46.
- Tan, R.X., Zou, W.X. 2001. Endophytes: a rich source of functional metabolites. *Natural Product Reports* 18(4): 448-459.
- Tanapichatsakul, C., Monggoot, S., Gentekaki, E., Pripdeevech, P. 2018. Antibacterial and antioxidant metabolites of *Diaporthe* spp. isolated from flowers of *Melodorum fruticosum*. *Current Microbiology* 75(4): 476-483.
- Turjaman, M., Hidayat, A. 2017. Agarwood-planted tree inventory in Indonesia. In: *IOP Conference Series: Earth and Environmental Science* 54: 012062.
- Turjaman, M., Hidayat, A., Santoso, E. 2016. Development of agarwood induction technology using endophytic fungi. In: Mohamed R (ed) *Agarwood: Science behind the fragrance*. Springer, Singapore, pp. 57-71.
- Tapwal, A., Pradhan, S., Chandra, S., Rashmi. 2016. Short communication: Antimycotic activity and phytochemical screening of fungal endophytes associated with *Santalum album*. *Nusantara Bioscience* 8(1): 14-17.
- Wang, Q.H., Peng, K., Tan, L.H., Dai, H.F. 2010. Aquilarin A, a new benzenoid derivative from the fresh stem of *Aquilaria sinensis*. *Molecules* 15(6): 4011-4016.
- Wetwitayaklung, P., Thavanapong, N., Charoenteeraboon, J. 2009. Chemical constituents and antimicrobial activity of essential oil and extracts of heartwood of *Aquilaria crassna* obtained from water distillation and supercritical fluid carbon dioxide extraction. *Silpakorn University Science and Technology Journal* 3(1): 25-33.
- Wheeler, K.A., Hocking, A.D. 1993. Interactions among xerophilic fungi associated with dried salted fish. *Journal of Applied Bacteriology* 74(2): 164-169.
- Yoshimi, N., Matsunaga, K., Katayama, M., Yamada, Y., Kuno, T., Qiao, Z., Hara, A., Yamahara, J., Mori, H. 2001. The inhibitory effects of mangiferin, a naturally occurring glucosylxanthone, in bowel carcinogenesis of male F344 rats. *Cancer Letters* 163(2): 163-170.
- Zhang, Z., Wei, J., Han, X., Liang, L., Yang, Y., Meng, H., Xu, Y., Gao, Z. 2014. The sesquiterpene biosynthesis and vessel-occlusion formation in stems of *Aquilaria sinensis*(lour.) gilg trees induced by wounding treatments without variation of microbial communities. *International Journal of Molecular Science* 15(2): 23589-23603.
- Zhou, W.N., White, J.F., Soares, M.A., Torres, M.S., Zhou, Z.P., Li, H.Y. 2015. Diversity of fungi associated with plants growing in geothermal ecosystems and evaluation of their capacities to enhance thermotolerance of host plants. *Journal of Plant Interactions* 10(1): 305-314.