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Research Article

Isolation, Identification and the Abilities of Fungi Associated with Agarwood from Bangka Belitung Island to Induce Agarwood Compounds

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ABSTRACT

Agarwood is one of the non-timber forest products that have high economic value. Agarwood is widely used to make incense, perfume and other products. Sapwood on agarwood is a group of secondary metabolites of agarwood plants that form a lump and have a certain color and aroma. The fragrant aroma of sapwood on agarwood is formed due to pathogenic infection of the agarwood tree. Until now, most studies of fungi forming sapwood are only oriented to virulent pathogenic fungi in nature and have never been reported to form hypovirulent agarwood. This study aimed to evaluate the potential of fungi originating from sapwood on agarwood especially those that are hypovirulent in inducing sesquiterpene compounds. This study included exploration, isolation, identification, and induction of fungi associated with sapwood on agarwood from four districts in the Bangka Belitung Islands Province. Hypovirulence test in cucumber sprouts and sesquiterpene induction test on agarwood plantlets was conducted in the laboratory. Based on the isolation results, 48 fungal isolates associated with sapwood on agarwood were found: fungi of genus *Fusarium, Trichoderma, Aspergillus, Penicillium, Curvularia, Peniophora*, and six isolates were unidentified. Based on the hypovirulence test on cucumber sprouts, 46 isolates of the fungus 46 isolates were virulent and 2 isolates were hypovirulent (isolates 4A and 17A). The induction of sesquiterpene compounds on the agarwood plant was employed using 5 sample isolates consisting of 4 virulent isolates (2A, 7A, 18A, and 25A) and 1 hypovirulent isolate (Isolate 4A). The results showed that hypovirulent isolates were able to produce sesquiterpenes even in small amounts compared with virulent isolates. Isolates produced many sesquiterpene compounds were isolates 18A (*Fusarium* sp.). Sesquiterpene compounds formed were pinene, terpineol, patchouli alcohol, trimethyl-naphthalene, beta-caryophyllene, camphor, eugenol, trimethyl-benzene, phenanthrene, citronella, eucalyptol, 4-hydroxy-4-methyl-2-pentanone. In this study also found fungi associated with sapwood on agarwood which had never been reported by previous researchers, *Peniophora* sp. (isolate 25A).

Keywords: hypovirulent, pathogen, *Peniophora* sp., sesquiterpene, *Trichoderma* sp.

INTRODUCTION

Agarwood is a superior commodity exported to Middle Eastern and European countries. Agarwood oil is a derivative product of agarwood commonly used in the production of perfume. There are many types of agarwood trees producing sapwood, such as *Aquillaria* genus (Ali *et al.,* 2012; Lancaster & Espinoza, 2012; Zhang *et al*., 2012; Azzarina *et al*., 2016). The fragrant aroma of sapwood on agarwood is formed due to the pathogenic infection (mostly from the fungal group) (Mohamed *et al*., 2014; Nurbaya *et al*., 2014), which is a secondary metabolite of sapwood and sapwood oil as antibacterial and anticancer (Gunasekera *et al*., 1981; Takemoto *et al.,* 2008; Wetwitayaklung *et al*., 2009; Chen *et al*., 2011; Ibrahim *et al*., 2011; Dahham *et al*., 2016). The sesquiterpene is a secondary metabolite of the high-quality sapwood on agarwood which gives a specific aroma to sapwood and agarwood oil (Hashim *et al*., 2014a). Sesquiterpenes compounds in sapwood on agarwood namely benzyl acetone, anisylaceton, guainen, palustrol, 8-epi-gama-eudesmol, α-guainene and alloaromadendrene oxide-1, agarospirol, γ-eudesmol, 3-phenyl-2-butanone, and α-cubebene (Hashim *et al.*, 2014b; Mohamed *et al*., 2014; Jayaraman & Mohamed, 2015; Sen *et al*., 2017).

Most of the research of sapwood on agarwood caused by fungi is focusing only to virulent pathogenic fungi (Wu *et al*., 2012a; Jong *et al*., 2014; Siburian *et al*., 2013; Leksonowati, 2016; Sen *et al*., 2017;

Faizal *et al*., 2017; Cui *et al*., 2013b; Zhang *et al*., 2014; Zhang *et al*., 2016) and there has been no research yet about hypovirulent pathogenic fungi caused sapwood on agarwood. Although the research on hypovirulent fungi has long been carried out and mostly explained the role of mycovirus in causing virulent fungi to be hypovirulent (Sneh *et al*., 1989; Chu *et al*., 2002 ; Castro *et al*., 2003; Prospero *et al*., 2006; Zhang *et al*., 2009; Yu *et al*., 2010; Baidyaroy *et al*., 2011; Darissa *et al*., 2011; Lee *et al*., 2011; Darissa *et al*., 2012; Wu *et al*., 2012b; Xiao *et al*., 2014; Ran *et al*., 2016).

The use of hypovirulent pathogen in agriculture has long been used as a biocontrol agent against plant diseases through their ability to induce plant resistance (Sneh, 1998; Robin *et al*., 2000; Muslim *et al*., 2003; Supriyanto *et al*., 2009; Supyani, 2017). Plants are infected by hypovirulent pathogen will induce the plant to form defense compounds against pathogenic infections. In addition, the use of hypovirulent fungi is environmentally friendly and does not cause symptoms of infection in other plants. Therefore, this study aimed to evaluate hypovirulent fungi cause sapwood on agarwood that could be used as a biological control agent to induce metabolic compounds secondary to agarwood plants, especially sesquiterpenes.

MATERIALS AND METHODS

Preparation ofTest Plants

Agarwood explants (*Aquillaria malaccensis*) were obtained from Tissue Culture Laboratory RSSNC (*Rumpin Seed Sources and Nursery Center*), Directorate of Forest Plant Seedling, Directorate General of Watershed and Protection Forest Management, Ministry of Environment and Forestry, Republic of Indonesia. Explant surface sterilization was carried out using 10% NaOCl solution for 10 minutes, then 5% NaOCl solution for 5 minutes and rinsed with sterile aqudest 2 times for 2 minutes. Explants *A. malaccensis* were cut 0.5 cm in length, then planted in 4.43 g/L of instant media Murashige Skoog (MS), added 30 g/L dextrose, 8 g/L agar, and pH 5.8. The bottle was covered with a heat-resistant plastic lid and closed with a plastic wrap to avoid contamination. These processes were carried out in an aseptic laminar airflow cabinet. Culture bottles containing agarwood plantlets were maintained by placing on culture shelves. The observation was conducted every two days, if the media or plantlets were contaminated, then they were transferred immediately to the new MS media.

The Sampling of Sapwood on Agarwood

Sapwood on agarwood was sampled from five villages located in four districts, Bangka Belitung Islands Province: Pangkal Buluh Village (South Bangka District), Lubuk Pabrik Village (Central Bangka District), Rukam Village and Sungai Buluh Village (West Bangka District), and Sempan Village (Bangka District). Sapwood on agarwood was collected from agarwood trees that grow in forests aged > 30 years old. The parameters observed were trunk diameter of each tree, elevation, and the coordinate using the Global Positioning System (GPS) (Figure 1). The samples were taken to the Non-Timber Forest Products Laboratory (HHBK), Regional Research and Development Planning Agency (BAPPELITBANGDA), Bangka Tengah District, for further isolation of the fungus associated with the sapwood on agarwood.

Isolation and Morphological Identification of Fungi Associated with Sapwood on Agarwood

Fungi isolation was carried out using direct isolation techniques from the agarwood tissue formed sapwood on agarwood which collected from the area between the healthy and infected tissue because the high activity of fungi is present in this area. Sterilization of wood tissue surface was conducted using 10% NaOCl solution for 10 minutes, immersion in 70% ethanol solution for 1 minute, rinsed with sterile distilled water 2 times, dried on sterile filter paper, and grown on 39 g/L Potato Dextrose Agar (PDA). Fungal colonies grow from pieces of agarwood were observed daily for one week and then subcultured onto new PDA. Identification of fungi associated with sapwood on agarwood was based on colony morphology, conidiophore, and conidiospore according to *Illustrated Genera of Imperfect Fungi* (Barnett & Hunter, 1998), *The Fusarium Laboratory Manual* (Leslie & Summerell, 2006), and *Introductory Mycology* (Wiley & Sons, 1960).

Hipovirulence Test

This hypovirulence test was conducted using cucumber sprouts to find out the hypovirulent fungi from the isolation test. The hypovirulence test used

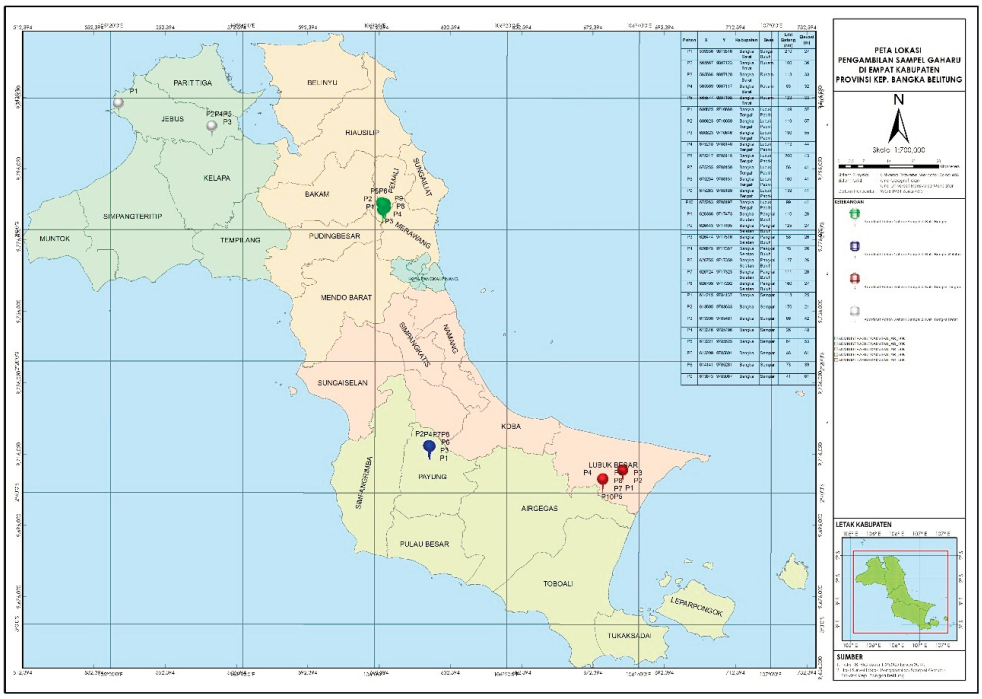

Figure 1. Location of the agarwood trees was sampled in the Bangka Belitung Islands Province (colored marker showing sample trees)

local varieties of cucumber plants, *Dawuan Kasokandel*, as an indicator plant using the method of Ichielevich-Auster *et al*. (1985) in Juan-Abgona *et al*., (1996). Cucumber seeds were disinfected with 70% ethanol for 1 minute, soaked 30 minutes in 2% sodium hypochlorite and Tween 20 solution with a concentration of 0.5 mL/L, and washed with sterile water 3 times. The seeds were germinated in a petri dish and incubated 2 days at room temperature, then transferred to water agar (2%) in a petri dish and grown 2-3 days at room temperature. Each fungi colony isolated (6 days old and 8 mm in diameter) was placed in the middle of the cucumber hypocotyl. For controls, cucumber plants were grown without inoculated by fungi. The treatment has 3 replicates and observations were conducted for 14 days by observing the Disease Severity Index (DSI) according to Cardoso and Echandi (1978) cit. Juan-Abgona *et al*. (1996). Isolates were categorized as hypovirulent if the value of DSI < 2. Completely Randomized Design was used to design the experiment and further analysis using Duncan's Multiple Range Test (DMRT). The DSI formula:

$$DSI = \frac{\sum N}{Z}$$

DSI = Disease Severity Index
N = value of the severity from each sample
Z = number of sample

The value of disease severity according to Cardoso & Echandi (1978) *cit.* Juan-Abgona *et al*. (1996):
0 = healthy, there is no infection in hypocotyl;
1 = one or two light brown spot < 0.25cm;
2 = light brown spot < 0.5 cm and wet area < 10% in hypocotyl;
3 = light to dark brown spot > 1.0 cm and then combined with other spots and wet areas 10% < X < 100% in hypocotyl (leaves have not wilt and hypocotyl is still growing up and white);
4 = hypocotyl fall down, leaves wilt and die.

Induction of sesquiterpene on agarwood planlet

After the hypovirulence test, five fungal isolates representing each district were selected for the induction of sesquiterpenes on agarwood planlet. These isolates were selected based on the percentage of the identified fungal genus, the hypovirulence test, and the fungal genus that had not been tested by previous researchers. The induction of sesquiterpenes in the agarwood plantlets aged 2 months was conducted by inoculating 1 cork drill bit (1 cm in diameter) from cultures of 5 fungal isolates associated with

sapwood on agarwood to the rooting area of the plantlets without making artificial wound to the roots. Each treatment was sampled for Gas Chromatography-Mass Spectrometry (GC-MS) analysis, 24 hours after inoculation (HAI), 48 HAI, and 72 HAI. All procedure was carried out aseptically in Laminar Air Flow Cabinet to avoid airborne fungi contamination in the plantlets.

Sesquiterpene extraction from plantlets was carried out using a method by Leksonowati (2016), only using a different solvent (absolute ethanol). The agarwood plantlets were crushed with a mortar, added 3 mL absolute ethanol solvent, and incubated 1 hour. The extraction was filtered with a Whatman filter paper No.42 (2.5 µm in pore diameter) then partitioned with a separating funnel to extract the filtrate. The filtrate was put into a small test tube (1 cm × 7.5 cm) and stored in a freezer of -20ºC before being analyzed by GC-MS. The same procedure was applied to extract plantlet tissue of control and treatments.

Analysis of Sesquiterpene by GC-MS

The filtrate was analyzed for sesquiterpene using Gas Chromatography-Mass Spectrometry method (Shimadzu type QP2010S Ultra brand) based on the method by Okudera & Ito (2009). GC-MS condition: AB-5MS capillary column (30 m × 0.25 mm), helium carrier gas, and solvent flow rate of 3.0 mL/minute. The column was programmed with an initial temperature of 50ºC and a final temperature of 300ºC. Sesquiterpenes formed were selected for analysis qualitatively. High-quality agarwood oil was used as a positive control, then analyzed by GC-MS in the same conditions as the samples and the negative control (agarwood plantlet was not inoculated with fungi). Sesquiterpene obtained from the sample was compared with the high-quality sesquiterpene and negative control.

RESULTS AND DISCUSSION

Isolation and Identification of Agarwood Fungi

Forty-eight isolates of fungi associated with sapwood on agarwood (consisting of 6 genera) were identified: *Fusarium, Trichoderma, Aspergillus, Penicillium, Peniophora, Curvularia*, and six isolates were unidentified (Table 1, Figure 2, and Figure 3). Although unidentified isolates actually produced spores and have been grown on the water agar media, yet they had no special structure, such as conidiophore of a particular fungus. The type of isolated fungus from the agarwood tree was diverse, however > 50% was dominated by the genus *Fusarium*. This result was similar to Nurbaya *et al*. (2014) reported that fungal isolates associated with sapwood in Nunukan District, North Kalimantan are fungi from the genus *Fusarium*. In addition, previous researchers also reported that the genus *Fusarium* is a predominantly fungal genus associated with sapwood on agarwood (Sangareswari *et al*., 2016; Mohamed *et al*., 2014).

In this study, not only *Fusarium* were found associated with sapwood on agarwood, but there were other genera such as *Penicillium, Aspergillus, Trichoderma, Curvularia,* and *Peniophora*. These results were similar with previous studies that reported several fungal genera commonly found associated with sapwood on agarwood: *Melanotus, Paeacremonium, Paraconithyrium, Fusarium, Chaetomium, Botryosphaeria, Aspergillus, Lasidiplodia, Penicillium, Xylaria, Curvularia, Trichoderma, Rhizopus, Clizospus, Cladosporium*, and *Cuninghamella* (Qi *et al*., 2005; Tamuli *et al*., 2011; Wu *et al*., 2012a; Cui *et al*., 2013a; Premalatha & Kalra, 2013; Siburian *et al*., 2013; Jong *et al*., 2014; Wen-Jian *et al*., 2014; Leksonowati, 2016; Sangareswari *et al*., 2016; Chhipa & Kaushik, 2017; Faizal *et al*., 2017; Sen *et al*., 2017; Zhang *et al*., 2017). This study also found a genus associated with sapwood that had not been previously reported: *Peniophora*. *Peniophora* sp. is a saprotrophic fungus causes white rot in plants (Lambevska *et al*., 2013). This fungus is widely used as a decomposing agent for its ability to convert organic into inorganic compounds.

Hypovirulence Test

From 48 fungal isolates were found associated with sapwood on agarwood, 2 isolates of *Trichoderma* sp. (4A and 17A) were hypovirulent, due to their low virulence level (DSI < 2) (Table 2). The low DSI value showed that the fungus is a hypovirulent (Juan-Abgona *et al*., 1996). Hypovirulent fungi have a low virulence level to infect plants, yet they can develop along with plant growth. These fungi could be used as antagonistic agents or PGPF (Plant Growth-Promoting Fungi). Supriyanto *et al*. (2009) conducted research using 42 isolates and found 28 hypovirulent isolates, then among the 28 isolates, only 2 isolates were PGPF.

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Table 1. Exploration and isolation of fungi associated with sapwood on agarwood in the Province of Bangka Belitung Islands

No.	District	Village	Tree	Trunk Diameter (cm)	Elevation (mdpl)	GPS coordinate		Isolate Code	Identification Result
						South	East		
1	South Bangka	Pangkal Buluh	P1	110	26	2º33'20.10"	106º08'12.00"	1A	*Fusarium* sp.
2		Pangkal Buluh	P2	125	27	2º33'19.40"	106º08'14.50"	1B	*Fusarium* sp.
3		Pangkal Buluh	P2	125	27	2º33'19.40"	106º08'14.50"	2A	*Fusarium* sp.
4		Pangkal Buluh	P2	125	27	2º33'19.40"	106º08'14.50"	2B	*Fusarium* sp.
5		Pangkal Buluh	P3	58	26	2º33'18.70"	106º08'15.50"	3A	*Trichoderma* sp.
6		Pangkal Buluh	P4	78	26	2º33'23.90"	106º08'22.00"	3B	*Fusarium* sp.
7		Pangkal Buluh	P6	127	26	2º33'24.10"	106º08'24.60"	4A	*Trichoderma* sp.
8		Pangkal Buluh	P7	111	26	2º33'25.00"	106º08'23.60"	4B	*Fusarium* sp.
9		Pangkal Buluh	P7	111	26	2º33'25.00"	106º08'23.60"	5A	*Fusarium* sp.
10		Pangkal Buluh	P8	160	27	2º33'26.00"	106º08'23.10"	5B	*Fusarium* sp.
11		Pangkal Buluh	P8	160	27	2º33'26.00"	106º08'23.10"	6A	*Aspergillus* sp.
12		Pangkal Buluh	P8	160	27	2º33'26.00"	106º08'23.10"	6B	Unidentified
13	Central Bangka	Lubuk Pabrik	P1	149	57	2º37'00.20"	106º37'35.30"	7A	*Fusarium* sp.
14		Lubuk Pabrik	P1	149	57	2º37'00.20"	106º37'35.30"	7B	*Fusarium* sp.
15		Lubuk Pabrik	P2	119	57	2º37'00.27"	106º37'35.31"	8A	*Fusarium* sp.
16		Lubuk Pabrik	P2	119	57	2º37'00.27"	106º37'35.31"	8B	*Fusarium* sp.
17		Lubuk Pabrik	P2	119	57	2º37'00.27"	106º37'35.31"	9A	*Fusarium* sp.
18		Lubuk Pabrik	P3	190	55	2º37'00.41"	106º37'35.19"	9B	*Trichoderma* sp.
19		Lubuk Pabrik	P4	113	44	2º38'22.29"	106º34'33.98"	10A	*Fusarium* sp.
20		Lubuk Pabrik	P4	113	44	2º38'22.29"	106º34'33.98"	10B	*Fusarium* sp.
21		Lubuk Pabrik	P4	113	44	2º38'22.29"	106º34'33.98"	11A	*Fusarium* sp.
22		Lubuk Pabrik	P5	200	43	2º38'22.16"	106º34'34.84"	11B	*Penicillium* sp.
23		Lubuk Pabrik	P7	56	41	2º38'22.02"	106º34'35.16"	12A	*Trichoderma* sp.
24		Lubuk Pabrik	P7	56	41	2º38'22.02"	106º34'35.16"	12B	*Trichoderma* sp.
25		Lubuk Pabrik	P8	160	41	2º38'22.01"	106º34'35.23"	13A	*Fusarium* sp.
26		Lubuk Pabrik	P8	160	41	2º38'22.01"	106º34'35.23"	13B	*Fusarium* sp.
27		Lubuk Pabrik	P8	160	41	2º38'22.01"	106º34'35.23"	14A	Unidentified
28		Lubuk Pabrik	P9	139	41	2º38'20.70"	106º34'35.41"	14B	Unidentified
29		Lubuk Pabrik	P10	99	41	2º38'20.43"	106º34'35.41"	15A	*Fusarium* sp.

Table 1. Exploration and isolation of fungi associated with sapwood on agarwood in the Province of Bangka Belitung Islands (continued)

No.	District	Village	Tree	Trunk Diameter (cm)	Elevation (mdpl)	GPS coordinate		Isolate Code	Identification Result
						South	East		
30	West Bangka	Sungai Buluh	P1	210	27	1°41'13.71"	105°21'13.31"	16A	*Penicillium* sp.
31		Sungai Buluh	P1	210	27	1°41'13.71"	105°21'13.31"	16B	*Trichoderma* sp.
32		Rukam	P2	100	36	1°44'42.05"	105°35'21.66"	17A	*Trichoderma* sp.
33		Rukam	P2	100	36	1°44'42.05"	105°35'21.66"	17B	*Trichoderma* sp.
34		Rukam	P3	113	33	1°44'41.55"	105°35'22.15"	18A	*Fusarium* sp.
35		Rukam	P4	83	32	1°44'41.92"	105°35'22.22"	18B	*Penicillium* sp.
36		Rukam	P5	123	33	1°44'42.20"	105°35'22.50"	19A	*Aspergillus* sp.
37		Rukam	P5	123	33	1°44'42.20"	105°35'22.50"	19B	Unidentified
38		Rukam	P5	123	33	1°44'42.20"	105°35'22.50"	20A	*Curvularia* sp.
39		Rukam	P5	123	33	1°44'42.20"	105°35'22.50"	20B	Unidentified
40	Bangka	Sempan	P1	113	25	1°57'09.60"	106°01'37.08"	21A	*Fusarium* sp.
41		Sempan	P2	170	21	1°57'26.00"	106°01'14.10"	21B	*Fusarium* sp.
42		Sempan	P2	170	21	1°57'26.00"	106°01'14.10"	22A	Unidentified
43		Sempan	P3	66	42	1°56'25.80"	106°01'10.33"	22B	*Fusarium* sp.
44		Sempan	P4	26	48	1°56'25.34"	106°01'07.92"	23A	*Penicillium* sp.
45		Sempan	P5	64	53	1°56'24.39"	106°01'04.88"	23B	*Fusarium* sp.
46		Sempan	P6	46	61	1°56'21.90"	106°01'04.44"	24A	*Fusarium* sp.
47		Sempan	P8	73	39	1°56'32.31"	106°01'34.63"	24B	*Fusarium* sp.
48		Sempan	P9	41	61	1°56'39.29"	106°01'19.51"	25A	*Peniophora* sp.

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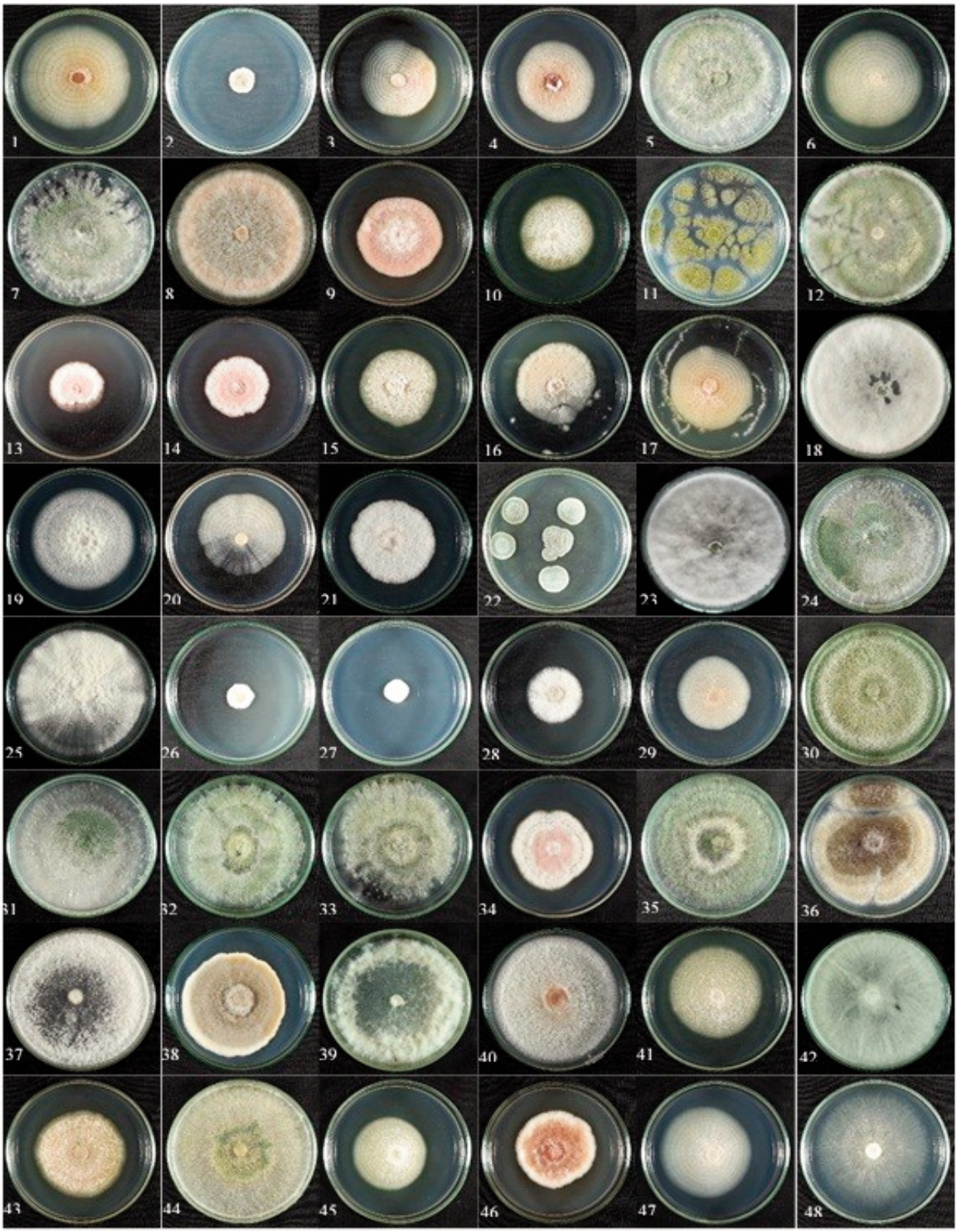

Figure 2. Colony morphology of the 48 agarwood isolates aged 9 days on PDA media, *Fusarium* sp. (number 1, 2, 3, 4, 6, 8, 9, 10, 13, 14, 15, 16, 17, 19, 20, 21, 25, 26, 29, 34, 40,41, 43, 45, 46 & 47), *Trichoderma* sp. (5, 7, 18, 23, 24, 31, 32 & 33), *Aspergillus* sp. (11 & 36), *Penicillium* sp. (22, 30, 35 & 44), *Peniophora* sp. (48), *Curvularia* sp. (38), and unidentified (12, 27, 28, 37, 39 & 42)

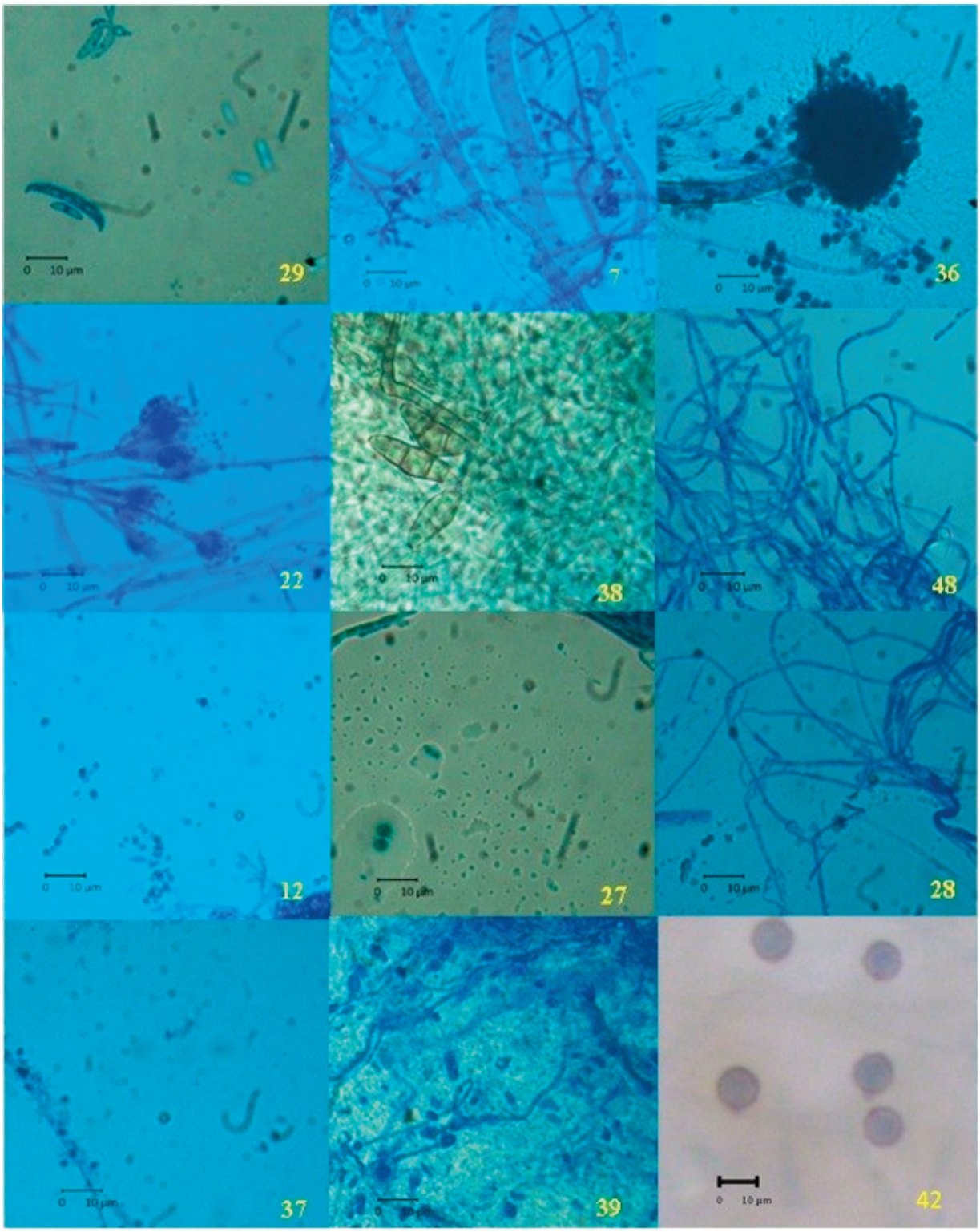

Figure 3. Conidium morphology of 6 genera agarwood fungi: *Fusarium* (29); *Trichoderma* (7); *Aspergillus* (36); *Penicillium* (22); *Curvularia* (38); *Peniophora* (48); and unidentified (12, 27, 28; 37, 39, & 42)

Induction of Sesquiterpene on Agarwood Planlet

Five isolates representing four districts were selected: 4 virulent isolates (2A, 7A, 18A, and 25A) and 1 hypovirulent isolate (4A). Isolates 2A, 7A, and 18A are *Fusarium* sp. (from South Bangka, Central Bangka, and West Bangka Districts), 25A is *Peniophora* sp. (from Bangka District), and 4A is *Trichoderma* sp. (from South Bangka District). From the induction test, 4 virulent isolates showed wilt symptom in the agarwood plantlets starting from 24 hours after inoculation (HAI), whereas no wilt symptoms observed from the hypovirulent isolates. The GC-MS analysis of the extraction of agarwood plantlets conducted in the next stage would be proven the result of the induction test for agarwood compounds.

GC-MS analysis showed that the compounds always appear in each treatment are decane and their derivatives, decanoic acid and its derivatives, naphthalene and its derivatives, patchouli alcohol, terpineol-4, benzene, and their derivatives, and 2-pentanone-4-hydroxy-4-methyl (Table 3). These compounds are produced through the mechanism of Induced Systemic Resistance (ISR) which is characterized by the presence of jasmonate and ethylene compounds. This result was similar with previous studies stated that methyl jasmonate acts as a receptor in the synthesis of sesquiterpene compounds (Xu *et al*., 2013c; Xu *et al*., 2013b; Liao *et al*., 2015). Thus the formation of this compound is through the mevalonate pathway. In addition, Xu *et al*. (2014) also found 3 enzymes of 1-deoxy-D-xylulose-5-phosphate synthase (DXS), i.e. AsDXS1, AsDXS2, and AsDXS3 as a precursor of the cytolytic mevalonate pathway.

In this study, jasmonate was still formed for 24 HAI. This result was different from previous studies reported that methyl jasmonate formed for 12 HAI (Kumeta & Ito, 2010; Azzarina *et al*., 2016b). Most of the sesquiterpene compounds formed could be expressed in the stem tissue of sapwood. As reported by Liao *et al*. (2015) that the AsCol1 (Coronatine-intensive protein 1) gene was mostly expressed in the tissue of the *A. sinensis* agarwood. Several compounds were found in each treatment at intervals of 72 HAI: dodecane, decanoic acid, and naphthalene (Table 4). Some of these compounds are phytoalexin produced by plants as a defense mechanism against pathogenic infections and toxic to pathogens.

Table 2. Disease severity index (DSI) value of 48 fungal isolates associated with sapwood on agarwood on the cucumber plant

No.	Isolate	Species	DSI
1	1A	*Fusarium* sp.	4b
2	1B	*Fusarium* sp.	4b
3	2A	*Fusarium* sp.	4b
4	2B	*Fusarium* sp.	4b
5	3A	*Trichoderma* sp.	4b
6	3B	*Fusarium* sp.	4b
7	4A	*Trichoderma* sp.	1.3a
8	4B	*Fusarium* sp.	4b
9	5A	*Fusarium* sp.	4b
10	5B	*Fusarium* sp.	4b
11	6A	*Aspergillus* sp.	4b
12	6B	Unidentified	4b
13	7A	*Fusarium* sp.	4b
14	7B	*Fusarium* sp.	4b
15	8A	*Fusarium* sp.	4b
16	8B	*Fusarium* sp.	4b
17	9A	*Fusarium* sp.	4b
18	9B	*Trichoderma* sp.	4b
19	10A	*Fusarium* sp.	4b
20	10B	*Fusarium* sp.	4b
21	11A	*Fusarium* sp.	4b
22	11B	*Penicillium* sp.	4b
23	12A	*Trichoderma* sp.	2.7b
24	12B	*Trichoderma* sp.	4b
25	13A	*Fusarium* sp.	4b
26	13B	*Fusarium* sp.	4b
27	14A	Unidentified	4b
28	14B	Unidentified	4b
29	15A	*Fusarium* sp.	4b
30	16A	*Penicillium* sp.	4b
31	16B	*Trichoderma* sp.	4b
32	17A	*Trichoderma* sp.	1.3a
33	17B	*Trichoderma* sp.	4b
34	18A	*Fusarium* sp.	4b
35	18B	*Penicillium* sp.	4b
36	19A	*Aspergillus* sp.	3b
37	19B	Unidentified	4b
38	20A	*Curvularia* sp.	4b
39	20B	Unidentified	2.7b
40	21A	*Fusarium* sp.	4b
41	21B	*Fusarium* sp.	4b
42	22A	Unidentified	4b
43	22B	*Fusarium* sp.	4b
44	23A	*Penicillium* sp.	4b
45	23B	*Fusarium* sp.	4b
46	24A	*Fusarium* sp.	4b
47	24B	*Fusarium* sp.	4b
48	25A	*Peniophora* sp.	3b
49	Control	Without treatment	0

Remarks: *)Values followed by the same letter were not significantly different according to DMRT at 5%.

Table 3. The GC-MS analysis of identified compounds in the treatment of five isolates

No.	Isolate 2A (*Fusarium* sp.)	Isolate 4A (*Trichoderma* sp.)	Isolate 7A (*Fusarium* sp.)	Isolate 18A (*Fusarium* sp.)	Isolate 25A (*Peniophora* sp.)
1	Decane	Dodecane	Dodecane	Dodecane	Doddecane
2	Tridecane	Gamma-terpinene	1,4,6-trimethyl-naphthalene	Pathcouli alcohol	2-pentanone, 4-hydroxy-4-methyl
3	Tetradecane	2-beta-pinene	1,5-dimethyl-naphthalene	Phenantrene	2,6-dimethyl-naphthalene
4	Decanoic acid	Camphene	Penantrene	Hexadecanoic acid	Hexadecanoic acid
5	Cyanic acid	Terpineol-4	Pathcouli alcohol	13-octadecanoic acid	Octadecanoic acid
6	Octene	Patchouli alcohol	Hexadecanoic acid	2-methyl-tetradecane	Nonanoic acid
7	2-heptadecanone	Pentadecanoic acid	Octadecanoic acid	Terpineol-4	
8	Dimethoxymethyl-silane	1,4,5-trimethyl-naphthalene	2-pentanone-4-hydroxy-4-methyl	1,4,5-trimethyl-naphthalene	Hexadecane
9	Acetic acid	1,4-dimethylnaphtalene	Nonane	2,6-dimethyl-napththalene	2-heptadecanone
10	1,2,3-trimethyl-benzene	Heptadecene	1-methyl-benzene	Tridecane	1-octadecyne
11	Alpha.pinene	Pentadecane	Decanoic acid	1,2,3-trymethylbenzene	Eicosyl-benzene
12		Hexadecanoic acid	3-octadecanone	Tridecane	1,2,4-trimethyl-benzene
13		Heptadecane	Hexadecane	Pentadecane	Patchouli alcohol
14		Octadecanoic acid	Camphor	Pentadecanoic acid	Eucaliptol
15		Eicosanoic acid		Hexadecane	
16		Decanoic acidtridecane		Octadecane	
17		Sabinene		Dimethoxymethyl-silene	
18		2-pentanone-4-hydroxy-4-methyl		Tetrakis(Ethoxy)-ethylene	
19				Trans-methyl dihydro jasmonate	
20				2-pentanone-4-hydroxy-4-methyl	
21				Camphor	
22				Citronella	

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Table 4. The GC-MS analysis of five isolates compared with high-quality agarwood oil compounds (standard)

No.	Compound	Agarwood oil high quality (standard)	Isolate 2A	Isolate 4A	Isolate 7A	Isolate 18A	Isolate 25A
1	Dodecane	Anti,Anti,Anti-3,3,6,6,9,9,12,12-Octamethyl-Pentacyclo[9.1.0.0(2,4).0(5,7).0(8,10)]Dodecane	+++	++	+++	+++	+
2	Decanoic acid	-	++	+++	+++	++	++
3	Pinene	Beta-vetivenene	+	+	-	-	-
4	Terpineol	Elemol; 10-epi-gamma eudesmol	-	+	-	+	-
5	Patchouli alkohol	Cyclohexanemethanol, 4-ethenyl-alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-, 1R-(1.alpha.,3.alpha., 4.beta.)	-	++	+++	+	++
6	Trymethyl-Naphthalene	1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha., 7.alpha.,7a.alpha.,7b.alpha.)]-(CAS).beta.-Gurjunene	-	++	+	++	+
7	Beta-caryophyllene	Beta.-Caryophyllene	-	-	+	-	-
8	Camphor	-	-	-	+	+	+
9	Eugenol	Guaiol; (-)-spathulenol	-	-	+	+	-
10	Trimethylbenzene	2-Butanone, 4-phenyl- (CAS) Benzylacetone; cis/trans-7-bicyclo[4.1.0]hept-7-ylidene-bicyclo[4.1.0]heptane	+	-	+	+++	+

Dou & Zhou (2012) stated that the plants will stimulate phytohormones or phytoalexin to defend against the infection of pathogens. Pathogens isolated from sapwood on agarwood in this study were *Fusarium* sp. and *Peniophora* sp. The quantity of phytoalexin produced by plants fluctuates according to the level of infection of pathogens, such as naphthalene. In the treatment of isolates 18A (*Fusarium* sp.), naphthalene was produced in a large amount for 24 HAI, then at 48 HAI, this compound was not formed because the pathogen has been localized by plants. However, at 72 HAI this compound began to form again (data not shown). It is suspected that pathogens may tolerate the phytoalexin produced by agarwood planlets.

Five isolates were able to form sesquiterpene with varying in the amount and type. From five isolates tested, 18A formed the most compounds, while 2A formed the fewest compounds even though they were pathogens from the same genus, *Fusarium* (Table 4). This result showed that the genetic factors in isolates have an important role to produce sesquiterpene of agarwood plants. The ability of *Fusarium* to induce sesquiterpene was reported by Faizal *et al*. (2017) that 9 sesquiterpenes and aromatic compounds were found after inoculated with *Fusarium* (from Gorontalo) to the agarwood tree *A. malaccensis*. Furthermore, besides the virulent isolates, hypovirulent isolate such as 4A (*Trichoderma* sp.) was able to form sesquiterpene similar to those in high quality of sapwood oil. This finding was the new result and has not been reported by previous studies. The results of GC-MS analysis showed variations in sesquiterpene compounds formed. The dodecane and decanoic acid sesquiterpene compounds are formed by all isolates. However, patchouli alcohol and trimethyl-1-naphthalene compounds are formed by hypovirulent isolate (4A) and three other pathogenic isolates (7A, 18A, and 25A), while 2A did not form these compounds. In addition, the terpineol sesquiterpene, included in the elemol and 10-epi-γ-eudesmol (high-quality agarwood oil) were able to be induced by hypovirulent isolate (4A) and pathogenic isolate (18A) while the other three isolates did not form these compounds. These results were similar with previous studies reported that elemol and 10-epi-γ-eudesmol are the high quality sesquiterpenes of agarwood (Sibuarian *et al*., 2013; Ismail *et al*., 2013; Mohamed *et al*., 2014; Sen *et al*., 2017).

This study also found a new finding on fungi that are able to induce sesquiterpene in agarwood plants, *Peniophora* sp. This fungus has never been studied by other researchers in inducing sesquiterpene in agarwood plants. Furthermore, there have been no reports of other researchers mentioning sesquiterpene produced by agarwood plants. Therefore, we suspect that sesquiterpene is produced for the uniqueness of *Peniophora* sp. in forming various kinds of enzymes. Previous researches reported that *Peniophora* produces enzymes, such as lactase (Shankar & Nill, 2015); cellulase (Trinh *et al*., 2013b), endo-beta-1,4-glucanase (Trinh *et al*., 2013a), and ligninolytic (Machado *et al*., 2005). Based on the results of this study, we concluded that not only virulent isolates are capable of inducing sesquiterpene in agarwood plants, but also hypovirulent isolate, i.e. 4A isolate (*Trichoderma* sp.). Therefore, hypovirulent isolates have an important role to be developed as a biocontrol against pathogens by inducing agarwood plants to produce sesquiterpene, because they are also environmentally friendly and less probability to become pathogens.

CONCLUSION

Forty-eight agarwood fungi isolates consisting of six genera were identified: *Fusarium, Trichoderma, Aspergillus, Penicillium, Curvularia, Peniophora*, and six isolates were unidentified. From the 48 isolates, 46 isolates were virulent and 2 isolates were hypovirulent. Fungi associated with sapwood on agarwood was also found, which had never been previously reported, i.e. *Peniophora* sp. Besides virulent isolate, hypovirulent isolate (*Trichoderma* sp.) also produce sesquiterpene of sapwood on agarwood. The isolate most produced type of sesquiterpene was 18A isolate (*Fusarium* sp.).

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