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ORIGINAL ARTICLE

Formation of agarwood from *Aquilaria malaccensis* in response to inoculation of local strains of *Fusarium solani*

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Abstract
Key message **Agarwood formation in *Aquilaria malaccensis* could be artificially stimulated by fungal infection. Furthermore, *A. malaccensis* adapts to this infection by developing typical included phloem boundary within xylem tissues.**
Abstract Naturally synthesized agarwood requires a lengthy process of up to 30 years, which impedes its continuous production. Therefore, recent effort has been allocated to the elucidation of agarwood formation to stimulate its process rapidly. In this study, we artificially induced agarwood formation by injection and inoculation of cultivated *Aquilaria malaccensis* with four strains of *Fusarium solani* isolated from different places in Indonesia. The results showed that *A. malaccensis* responded differently upon wounding and fungal inoculations compared to healthy trees. All wounded and inoculated samples resulted in the formation of typical discoloration zone surrounding injection sites. Further anatomical observation revealed that both samples also developed included phloem structures in which resinous agarwood compounds were accumulated. Gas chromatography–mass spectrometry (GC–MS) analysis of the inoculated samples yielded some important agarwood compounds such as tridecanoic acid, α-santalol, and spathulenol, which were not present in both healthy controls and only wounded samples. Notably, one of the tested *F. solani*, strain Gorontalo displayed promising results as a candidate for artificially induced agarwood formation in *A. malaccensis* in terms of color, odor, and chemical constituents.

Keywords Agarwood · *Aquilaria malaccensis* · *Fusarium solani* · Included phloem · Sesquiterpenoid

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Introduction

Aquilaria malaccensis is a tropical plant species widely known as agarwood-producing species from the family Thymelaeaceae. This species is native to South and Southeast Asia with Indonesia and Malaysia being the two major sources of agarwood (Persoon 2007). However, the overexploitation of natural agarwood has hitherto affected the availability of agarwood-producing species in their natural habitats. In November 1994, all *Aquilaria* species have been listed in Appendix II of CITES (the Convention on the International Trade in Endangered Species of Wild Flora and Fauna) to prevent its excessive exploitation and to regulate its trade (CITES 2004).

Agarwood is a resinous compound produced by plants as a response to physical wound as well as pathogen attacks (Karlinasari et al. 2015). Furthermore, agarwood is a highly commercial non-timber forest product due to its important role in fragrances, aromatherapy, medicines, and religious activities (Chen et al. 2012). Over the past few decades, more than 150 compounds have been identified as constituents of agarwood, which comprise mixtures of chromones, volatile aromatic compounds and sesquiterpenoids (Naef 2011).

Due to the significant increase in agarwood demand and its high price, some efforts have been made to stimulate agarwood production artificially as well as to drive a faster process of its formation. Furthermore, numerous approaches have been developed to find the most efficient technique of agarwood production to meet the high demand, and at the same time, it could decrease exploitation of the remaining trees in the natural habitat (Jayaraman et al. 2014; Li et al. 2015; Liu et al. 2013; Zhang et al. 2014a, b). Agarwood farmers in different Asian countries have tried several wounding methods to produce agarwood, including chopping, nailing, holing and trunk breaking. These methods often take a long time, with generally inadequate and low quality in the agarwood production (Li et al. 2015; Liu et al. 2013; Yagura et al. 2005; Zhang et al. 2014a). In addition, chemical inducers such as sodium chloride and hydrogen peroxide have also been applied in many countries (Chen et al. 2011; Zhang et al. 2014b). However, this approach is becoming less preferable due to side effects of the chemicals which are harmful to the environment.

Previous studies showed that different induction methods resulted in different agarwood qualities. Some reported that essential oils originated from agarwood induced by nailing and holing of *Aquilaria* stem contain high number of major sesquiterpenes and aromatic groups, while those induced by trunk breaking contain high amount of fatty acids (Lin et al. 2010). In contrast, Tamuli et al. (2005) reported that among the chemical constituents found, fatty acids predominantly exist in both healthy and fungal-inoculated woods. This present study addressed the comparison of chemical constituents in the extracts of both wounded and fungal-inoculated stems of *A. malaccensis* to the chemical profiles of healthy plants.

At present, the exact mechanism underlying the formation of agarwood in the tree remains unclear. Previous reports have shown that wounding played significant role in agarwood formation (Xu et al. 2013; Zhang et al. 2014a). On the other hand, the capacity of fungi in eliciting the production of agarwood cannot be excluded, as numbers of fungi species and/or strains have been isolated from *Aquilaria* spp. (Jong et al. 2014; Subehan et al. 2005; Wong et al. 2015). The common fungi reported to infect *Aquilaria* spp. include *Botryosphaeria*, *Colletotrichum gloeosporioides*, *Trichoderma* sp., *Lasiodiplodia* sp., and *Fusarium* spp. (Mohamed et al. 2010; Premalatha and Kalra 2013; Tian et al. 2013).

In Indonesia, general studies on fungal–*Aquilaria* interaction carried out in different regions had already isolated and identified different species of *Fusarium* including *F. xylaroides*, *F. falciforme*, *F. oxysporum*, *F. ambrosium*, and *F. solani*. We subsequently draw our focus on *F. solani* since this fungus is associated with *Aquilaria* spp. very dominantly. This fungus was also reported to induce agarwood formation more effectively when compared with others (Sitepu et al. 2011). In this report, four identified strains of *F. solani*, namely GSL1–4 with their respective origins from Gorontalo Province (Celebes Island), Jambi Province (Sumatra Island), Papua Province (Papua Island) and Singkep Island (Riau Archipelago Province) were inoculated on cultivated *A. malaccensis* trees to induce the formation of agarwood. The resulting plant responses upon fungal infection and/or wounding, including anatomical features and biochemical changes were then analyzed.

Materials and methods

Plant materials and growth condition

Aquilaria malaccensis trees were cultivated in Block 44, Kalirajut Resort, Kebasen District at 90 m above sea level, East Banyumas, Central Java Province (7°31.003′ to 07°31.014′S and 109°12.089′ to 109°12.105′E), Indonesia and belong to Indonesia state forestry company (PERHUTANI). Thirty plants used in this study were randomly selected based on diameter at breast height (DBH) between 15 and 18 cm (approximately 10 years old) with height range between 6 and 8 m (Karlinasari et al. 2015; Sitepu et al. 2011). These plants were then divided into three treatment groups: healthy control plants, wounded and inoculated plants with each group comprising ten plants.

Fungal inoculation

Fusarium solani strains GSL1–4 were previously isolated from wild *A. malaccensis* grown in different places in Indonesia by the Forestry Research, Development, and Innovation Agency (FORDA), Ministry of Environment and Forestry, Republic of Indonesia. Those strains are commercially available in the form of juice-liquid and are ready to use. Prior to inoculation, all treated stems were drilled perpendicular at breast height; the hole diameter was 0.3 mm and the inward depth was one-third (1/3) of the stem diameter. Additional holes were made vertically 20 cm above and under breast height and horizontally 10 cm from the vertical holes. One mL of fungal strains was injected in each hole and subsequently covered by plasticine to avoid infection by any other cause. As for wounded samples, the stems were only drilled without further fungal inoculation (Sitepu et al. 2011).

Measurement of discoloration zone

Bark was removed from the wounded and/or inoculated stem area to ensure proper observation on the discoloration

zone. The extended discoloration zone at both upper and lower part of wound site was corresponded with ellipsoidal shape; hence, this zone was measured in accordance with the measurement of ellipse area:

$$A_{\text{ellipse}} = \frac{1}{2}\pi ab,$$

where a and b are the ellipse's major and minor axes, respectively.

The obtained data were then analyzed using one-way ANOVA followed by Duncan's multiple range test. The statistical analysis was performed at the level of P value less than 0.05 using SPSS 18.0 (SPSS Inc. USA).

Olfactory test

Agarwood is a fragrant resinous heartwood with typical scent that is attributed to its volatile constituents. Therefore, in this study, we conducted olfactory test to evaluate the odor of wood samples. The wood samples isolated from wounded site were cut off into small pieces and subsequently crushed with a warring blender to obtain 2 g of wood powder. A total of 57 independent respondents were subjected to this test and were asked to score the scent of random samples from 1 (barely smell) to 5 (very strong smell). Wood samples from healthy plants were used as control.

Anatomical observation

Wood tissue from wounded sites on *A. malaccensis* trunks was dissected and then fixated with a mixture of aquadest:ethanol:glycerin (1:1:3). The same treatment was applied on healthy plant samples. The wood tissue was softened by immersion on a porcelain dish incubated in boiling water for 2 h. The obtained wood sections were then embedded in paraffin and stained with a neutral red dye (0.01 % in aqueous solution pH 8.0) to check the formation of included phloem. The wood sections were mounted on microscopic slides and were examined under a reflected light microscope with a magnification range between 100 and 400×. Furthermore, the lactophenol cotton blue (LPCB) staining was used to detect the presence of remaining fungal hypha on wood samples (Leck 1999).

Sample extraction and GC–MS analysis

One gram of grounded samples was extracted with 10 mL ethyl acetate. The samples were subsequently incubated on a rotary shaker 100 rpm for 24 h and sequentially diluted until total volume of 30 mL. The incubation process allowed the cells to break up, thus easing the wood component to volatilize and improving the yield. The extracts were then centrifuged at 1000 rpm for 10 min. 0.5 mL of supernatant was isolated for further analysis with GC–MS (Fazila and Halim 2012; Yagura et al. 2003).

GC–MS analysis of these extracts was performed using a GC-17A (Shimadzu) and gas chromatograph interfaced to a mass spectrometer, MS QP 5050A equipped with a silica capillary column (30 m × 0.25 mm × 0.25 μm). Helium gas was used as the carrier gas at constant flow rate 1 mL/min and an injection volume of 2 μL. The temperature was programmed from 50 °C with an increase of 15 °C/min to 280 °C. Total GC running time was 31 min. At least three independent samples from each group of treatment were subjected for GC–MS analysis. The relative amount (%) of each component was calculated by comparing its average peak area to the total areas. The resulting chromatograms were integrated and aligned according to their groups. Identification of the chemical components was based on the comparison of the calculation of their retention time and authentic mass spectral data with the existing Wiley MS libraries 2008.

Results

Formation of discoloration zone

By the first week after having been wounded and inoculated, discoloration zone started to develop surrounding the injection site on all trees, except on healthy plants. The zone was marked by dark area that tended to extend vertically to both upper and lower parts of the injection sites (Fig. 1). The development of these zones inferred that inoculation of strain GSL1 significantly yielded the largest area followed by strain GSL3, while the areas of strain GSL2 and GSL4 were not significantly different from that of wounded sample (Fig. 2). This result implied that GSL1 (strain Gorontalo) developed a more favorable agarwood compared to other treatments.

Microscopic observation

Agarwood is a secondary metabolite produced by the wood tissues of *Aquilaria* plants. Therefore, the secondary xylem tissue was the most interesting part of our study. Anatomical observation of xylem tissues under light microscopes clearly showed the cell structure of tracheid, ray parenchyma and their vessel elements (Fig. 3). Interestingly, both wounded and inoculated plants formed included phloem or secondary phloem within xylem tissue in which scented resinous matters were suspected to have accumulated. This indicated that plants might develop similar anatomically based defense response to wounding

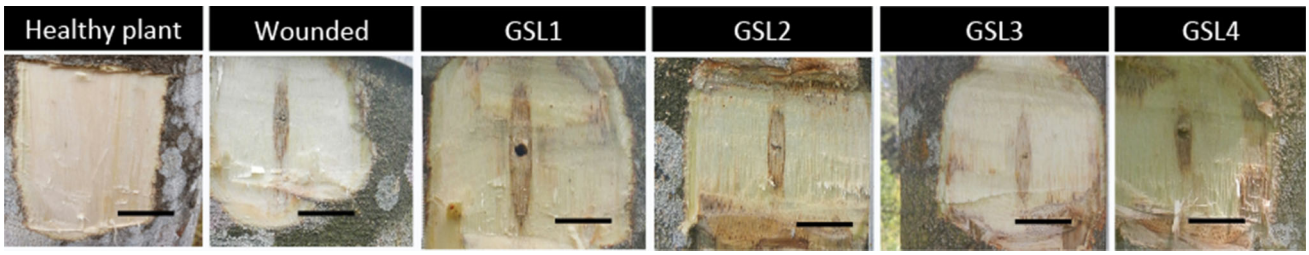

Fig. 1 Discoloration zone around injection site on *A. malaccensis* stems after inoculation with different strains of *F. solani* (pictures were taken 12 weeks after inoculation). *Bars* 2 cm

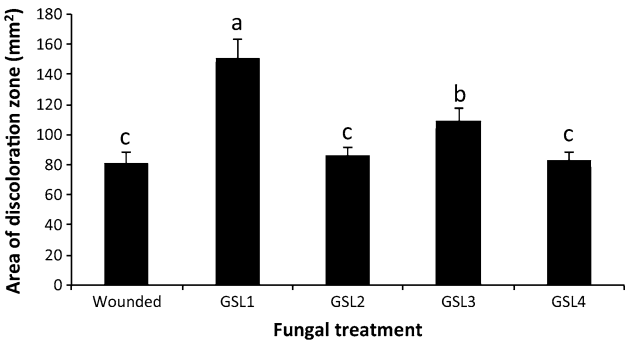

Fig. 2 Area of discoloration zone formed on *A. malaccensis* stems 12 weeks after inoculation. *Vertical bars* indicate the standard error of the mean of at least ten replicate experiments. Different letters indicate significant differences ($P < 0.05$) according to Duncan's test

and fungal inoculation. Furthermore, we also confirmed the presence of fungal hyphae on the harvested wood tissue as displayed in Fig. 4.

Olfactory test

Currently, one of the standards to qualify the grade of agarwood is the fragrance evolving from wood samples. The wood samples from healthy plants have no specific odor, while the odor of wounded and inoculated samples was somewhat pleasant. Therefore, we qualitatively compared the odor of wounded and GSL-inoculated samples through a survey using an olfactory test. The result showed that wood inoculated with GSL1 (strain Gorontalo) has the strongest pleasant odor (Fig. 5). This also indicated that GSL1 induced a better quality of agarwood compared to other treatments.

GC–MS analysis and identification of compounds

Based on the preliminary results on wood sections and olfactory test, we further proceeded with only GSL1-inoculated samples to evaluate the response of *A. malaccensis* upon *F. solani* infection. Three independent wood samples from healthy, wounded, and GSL1-inoculated plants were profiled with gas chromatography to compare the constituents of agarwood induced by wounding and fungal inoculation with those of control healthy plants. Figure 6 depicts the chromatogram of an extract of GSL1-inoculated *A. malaccensis* and Table 1 gives an overview of detected agarwood compounds.

Both wounded and GSL1 samples seemed to accumulate similar agarwood substances. These include β-elemene, isoaromadendrene epoxide, aromadendrene oxide-(1), and aromadendrene oxide-(2), yet these compounds were found relatively much more abundant in GSL1-inoculated samples. Interestingly, the defining compounds such as α-santalol, spathulenol, tridecanoic acid, and stigmasterol which are the main agarwood constituents were present only in GSL1-inculated plants, indicating that *A. malaccensis* developed different agarwood formation in response

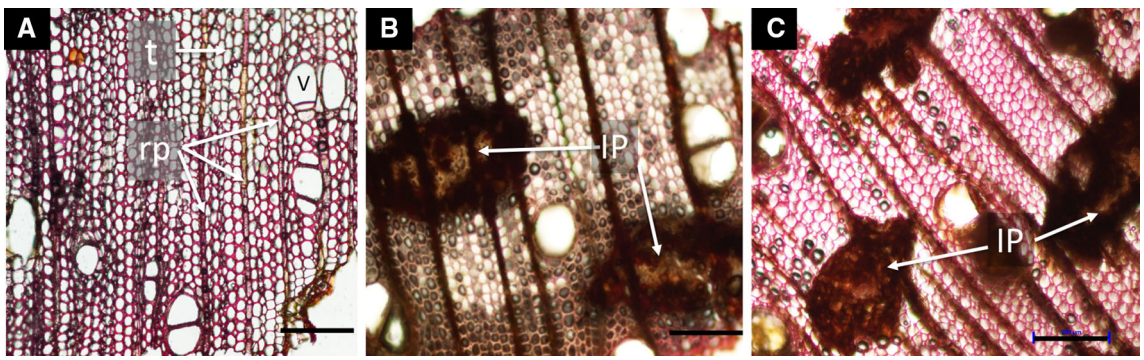

Fig. 3 Transversal section of wood tissue obtained from healthy (**a**), wounded (**b**), and inoculated plants (**c**) stained by neutral red dye. *v* Vessel element, *t* tracheid, *rp* ray parenchyma, *IP* included phloem. *Bars* 200 μM

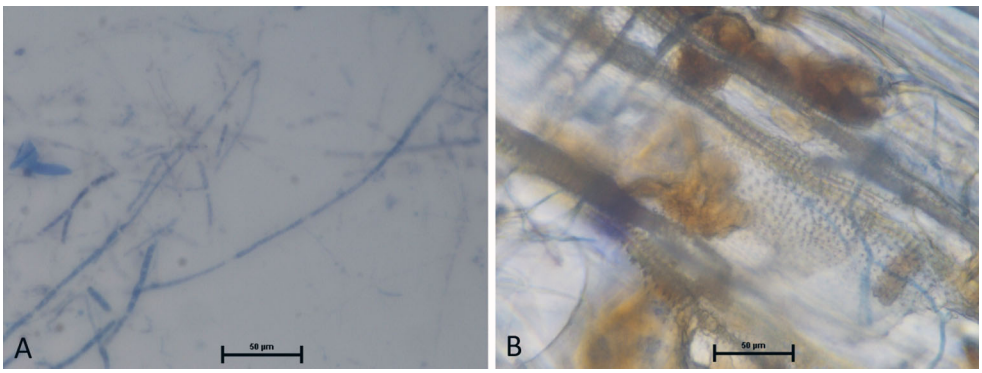

Fig. 4 Microscopic observation of fungal hypha obtained from GSL suspension (**a**) and the respective hypha, which infected *A. malaccensis* stem (**b**). *Bars* = 50 µM

Fig. 5 The odor level of different *A. malaccensis* samples based on olfactory test performed on 57 independent respondents. *1* Nearly odorless, *2* fair odor, *3* less strong odor, *4* strong odor, *5* very strong and pleasant odor

to wounding and fungal infection. Notably, nine discrete peaks corresponding to high-grade agarwood constituents were only detected in GSL1-inoculated and/or wounded samples and were absent in control healthy plants. In addition, we also detected benzyl benzoate as a major component in healthy plant samples, which was not present in both wounded and GSL1-inoculated samples.

Discussion

Plants normally synthesize a plethora of secondary metabolites as response against pathogen attacks or to survive under other biotic and abiotic stresses (Atkinson et al. 2015; Lambert et al. 2011; Sommano 2015). Production of resinous agarwood is also thought to be an example of these phenomena. Kumeta and Ito (2010) reported that the sesquiterpenes found in agarwood are also produced as phytoalexins under stress.

Despite its broad applications and high market demands, production of natural agarwood is currently limited by the availability of plant sources. Agarwood-producing plants are timber species from tropical region that require considerable time to grow and form resinous portions inside the wood only when affected by certain factors such as lightning strike, wind breaking, man-intended wound, insect attack or microbial invasion. Because of this, we opted to accelerate the agarwood production by infecting cultivated *A. malaccensis* with fungi that normally inhabit agarwood-producing trees. Therefore, four strains of *F. solani*, isolated from different places in Indonesia, were injected and inoculated into agarwood-producing species *A. malaccensis* trees.

The formation of agarwood in both wounded and inoculated *A. malaccensis* could be easily recognized by the presence of dark-brown area or discoloration zone surrounding the wounding site, which was not found in healthy plants. This dark zone typically extended from wound/injection site and intensifies its dark color after 3 months. This also explained that a period of stress was critical for agarwood formation in *A. malaccensis*. Similar study which suggested the importance of duration of stress was inoculation of *Chaetomium globosum* on *A. agallocha* which, after 1 month, showed no significant differences in oil compositions with that of healthy trees (Tamuli et al. 2005). Taken together, the difference in darkened area on *A. malaccensis* stems most likely depended on the wound level caused by the differences in the virulence abilities of fungal strains. By examining the infection development on the stem of *A. malaccensis* trees, it can be inferred that the GSL1 (strain Gorontalo) resulted in the largest infection. This implied that the virulence of strain GSL1 was presumably higher or adjusted better to the new place than that of the other strains.

Further investigation was carried out on wood tissues with darkened area to explain the correlation between

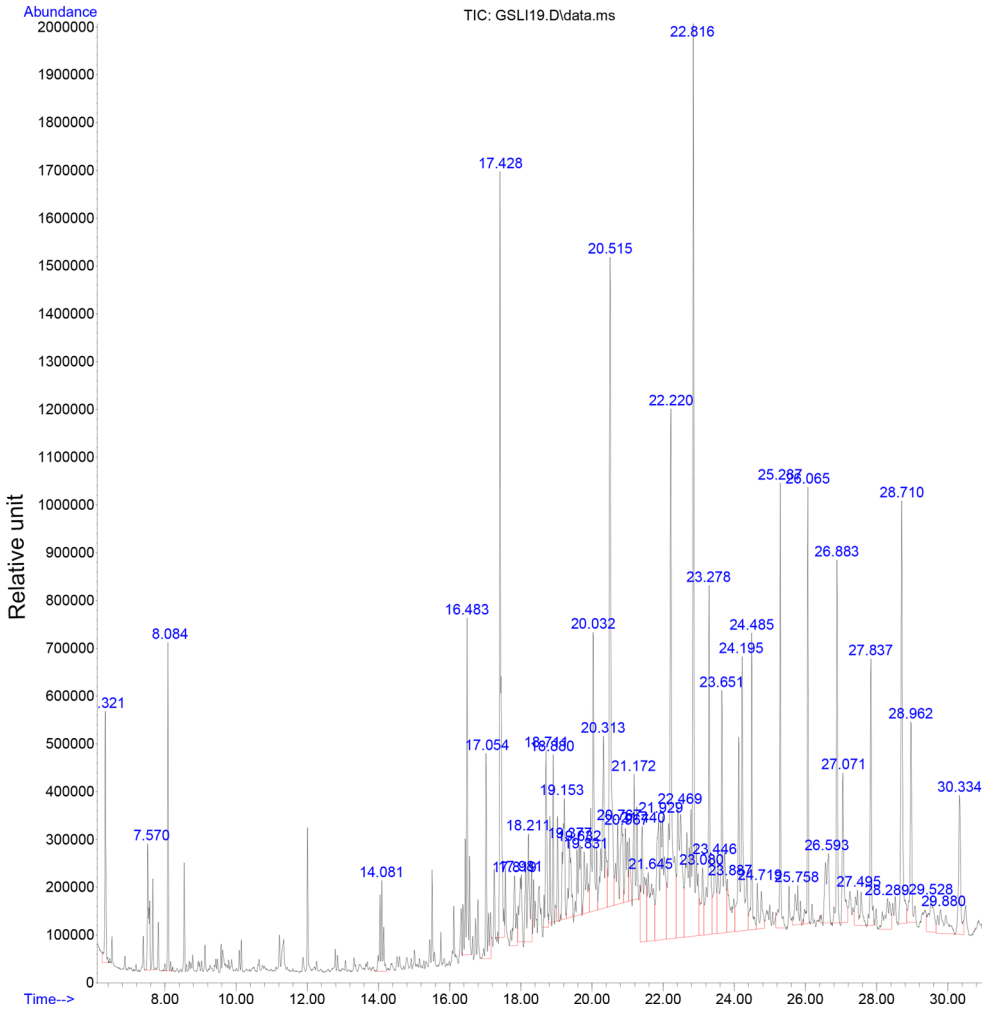

Fig. 6 GC-chromatogram of GSL1-inoculated plants

plant tissue organization and resin accumulation mechanism in agarwood-producing plants. This was in accordance with the study of resin-producing plants, such as conifers that secrete and accumulate their resinous compounds in the secondary xylem tissues (Hudgins and Franceschi 2004). Microscopic observation of the wood tissues revealed that *A. malaccensis* adapted to wounding and fungal infection by developing typical included phloem boundary within xylem tissues. This structure is functionally similar to the resin duct of conifers in terms of compartmentalization of the decayed wood upon wounding and pathogen–plant interactions (Blanchette 1992). Though, resinous agarwood produced by *Aquilaria* species was not exuded out of wood, but deposited and infiltrated in their included phloem strands as reported also in *A. agallocha* and *A. microcarpa* (Hasibuan et al. 2013; Rao and Dayal 1992). It can be concluded that *Aquilaria* species develops an exceptional modification from others in the production of barriers that effectively compartmentalize injuries and infections.

Agarwood is available in the market in various qualities, depending on the resinous content, color and aroma. It is, therefore, interesting to evaluate the agarwood quality of the treated *A. malaccensis* in this recent study. Generally, agarwood is recognized by typical sweet to pleasant fragrance exuded when the wood is burned. Here, we tested the odor of wood powder from wounded and fungal-treated samples. In addition to its darker discoloration, GSL1-treated woods also released the most pleasant fragrance among others. This corroborates with the previous findings that different artificial methods may result in different qualities of agarwood (Li et al. 2015; Naef 2011; Tamuli et al. 2005).

It is a common knowledge that plants respond to wounding and fungal attack by activating similar complex of regulatory mechanism to recognize and trigger defense responses (Cheong et al. 2002). This includes biosynthesis of secondary compounds that play important roles as toxic chemical agents in plants (Lange 2015). To accomplish our findings, we further characterized the treated wood samples using GC–MS analysis.

Table 1 Compounds detected from different samples of *A. malaccensis* by GC–MS

Compound		Retention time	Relative peak area (%)		
			Healthy plant	Wounded	GSL1 inoculation
Sesquiterpenes and aromatics					
1	α-Santalol	16.48	–	–	3.31
2	Cyclohexane,1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S(1α,2β,4β)]-(β-elemene)	17.43	–	1.25	4.74
3	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a α, 4aα, 7β, 7aβ, 7bα)]- [spathulenol]	18.71	–	–	1.22
4	Aromadendrene oxide-(2)	19.83	–	0.88	0.94
5	Isoaromadendrene epoxide	20.03	–	0.98	2.74
6	Aromadendrene oxide-(1)	22.81	–	0.92	8.01
Fatty acids and alkanes					
7	Tridecanoic acid	20.51	–	–	5.43
8	Oleic acid	22.22	2.52	6.41	5.33
Sterol					
9	Stigmasterol	27.26	–	–	1.08
Fenol					
10	Benzyl benzoate	18.60	11.64	–	–

– Not detected

A total of 50 compounds were identified in each sample ranging from hydrocarbons, phenols, fatty acids, terpenes (di-, tri-, and sesquiterpenes), and other compounds. Furthermore, different compositions were contributed to the relatively largest portion of total compounds in each chromatogram of all treatments. About 20 % of compounds of interest identified in GSL1 samples were aromatic and sesquiterpenes, which have been revealed to be the main active compounds of agarwood and contributed to its pleasant fragrance, such as α-santalol, spathulenol, β-elemene, aromadendrene oxide-(1), aromadendrene oxide-(2) and isoaromadendrene epoxide. In addition, a fatty acid, i.e., tridecanoic acid was abundantly present only in fungal-inoculated samples. Together with aromadendrene oxide-(1), these compounds were found as major constituents of agarwood in fungal-treated samples, comprising 13.44 % of the total compound. Interestingly, oleic acid as one of the main compounds that are normally present in agarwood essential oil was detected in all samples, yet it was found relatively more abundant in both wounded and fungal-inoculated plants.

Both wounded and inoculated samples of *A. malaccensis* contained mixture of sesquiterpenes and aromatic compounds in substantial amounts, which were not only associated with plant response upon stress treatment, but also contributed to agarwood fragrance. On the other hand, our results show that *A. malaccensis* may respond to pathogen attack differently from mechanical wounding. This is supported by the fact that fungal-inoculated samples produced more concentrated sesquiterpenes which were somewhat not present in wounded samples. Interestingly, benzyl benzoate was identified as a major component found in healthy plants, but absent in both wounded and inoculated samples. We suggest that this chemical serves as biosynthetic intermediate for other compounds such as benzoic acid and involves in early response upon stress treatments in plants as previously reported (Hilker and Meiners 2006; Schwab et al. 2008).

In conclusion, the artificial induction of agarwood by means of fungal inoculation has been proven to be a promising technique for agarwood formation. This technique drives the process much faster compared to physical–mechanical induction and is more environmentally friendly compared to chemical inducers. This report also provides a development on artificial induction as well as the elucidation of fungal–plant interaction on agarwood formation. Nevertheless, the developed approach in this study should be further optimized so that the technique will become more efficient, cost-effective, yet produces high-grade agarwood.

Author contribution statement AF, RRE, EAN, and IR designed the study, collected data, developed the methodology, performed the analysis, and wrote the manuscript. ES and MT collected data and developed the methodology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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