EFFECT OF INOCULATING ENDOPHYTIC FUNGI ON AGARWOOD FORMATION IN *AQUILARIA MALACCENSIS* LAM.*

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Abstract

The fourteen endophytic fungi were isolated from the healthy plant parts such as leaves, bark, wood and fruit of *Aquilaria malaccensis* Lam. The fourteen isolated fungal strains were tested for enzymatic activities such as cellulase, laccase, lipase, protease and pectinase. *Aspergillus* sp. (CM 2 and CM 11) showed cellulase, lipase and pectinase activities. *Curvularia* sp. (CM 6) showed laccase. *Aspergillus* sp. (CM 2 and CM 11) and *Curvularia* sp. (CM 6) were selected as the potential endophytic fungi for inducing the agarwood formation. The three strains of endophytic fungi such as *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and *Aspergillus* sp. (CM 11) were used as inoculation materials. The fungal inoculation was done in the stem of *Aquilaria malaccensis* Lam. using the syringe method. The induced agarwood was harvested after 3 months of inoculation. The inoculation of *Aspergillus* sp. (CM 11) showed better results in average agarwood yield (3.5 %) per tree. The average agarwood yield per tree treated with *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and Potato dextrose broth (Control) was showed 1.9 %, 0.7 % and 0.2 % respectively. The 2.53 % of agarospirol production was induced successfully using the fungi *Aspergillus* sp. (CM 11) within 3 months only, after inducing the infection. Therefore, *Aspergillus* sp. (CM 11) is an efficient and promising fungal strain for artificial agarwood production during commercial-scale cultivation of *Aquilaria malaccensis* Lam. which can lead to the ecology and ecological stabilities for communities in near future.

Keywords: isolation, endophytic fungi, enzymatic activity, inoculation, agarwood

Introduction

Agarwood is a resinous portion of *Aquilaria* trees, a genus belonging to the family Thymelaeaceae (Gao *et al*. 2019). Agarwood-producing trees are widely distributed in Asia, including India, Sri Lanka, Bangladesh, Bhutan, Myanmar, Laos, Vietnam, Cambodia, China, Brunei, Malaysia, Indonesia, Philippines, Thailand, and Papua New Guinea (Turjaman *et al*. 2016). The healthy wood of *Aquilaria* Lam. trees is white, soft, and without scented resins. The dark resinous material of *Aquilaria* is created as a response to natural injury by lightning strike, animal grazing, insect attack, and microbial invasion (Blanchette & Heuveling 2009).

The natural production of agarwood is rare and slow, as only 7-10 % of old trees can produce agarwood, resulting in high agarwood prices on the global market. Agarwood can fetch as much as US$ 100,000 per kilogram for superior pure wood material or US$ 100 per kilogram for low quality. The high economic and medicinal value of agarwood has increased market demand, leading to the overharvesting of agarwood resources and destruction of natural *Aquilaria* forests (Naef, 2011).

Agarwood oleoresin composed of sesquiterpene and chromone derivatives and their composition determines the quality or grade of agarwood in the market (Chhipa & Kaushik 2017). The oleoresin produced by the tree is a highly valuable component of incense, perfumes, drugs, stimulants, cardiac tonics, and carminatives (Mengling *et al*., 2005). The over-use of

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agarwood has seriously affected the natural resources of all *Aquilaria* species capable of producing agarwood, thus these endangered species listed in Appendix II of the Convention on Internal Trade in Endangered Species of Wild Fauna and Flora (CITES) since 2004 (Chen *et al*. 2011).

To protect the wild plant resources and to meet the demand for sustainable agarwood production, *Aquilaria* trees are now being planted in large area by using a variety of artificial cultivation mode (Lei *et al*. 2019). The three common induction techniques are physical, chemical, and biological (Arzen *et al.* 2019). Biological induction techniques involve the stimulation of the plant's immune response through artificial infection of "pure" or "mixed" fungal strains. The majority of fungi used for the inoculation are endophytes isolated from healthy plants or the infected tissue of agarwood-producing trees (Rasool & Mohamed 2016).

Along with the continuously decreasing supply of wild agarwood and in order to meet market demand, much effort will make to preserve natural *Aqualaria* species populations and to increase agarwood supply. Therefore, the study on the effect of inoculating the endophytic fungi to induce the agarwood formation is important to improve cultivation of *Aqualaria* species.

The general aim and objectives of this research is to earn income generation through sustainable and artificial induction technique. The specific aim and objectives of this research are to isolate and identify the endophytic fungi from healthy plant parts of *Aquilaria malaccensis* Lam., to study the enzymatic activities of endophytic fungi, to induce agarwood formation by inoculating with endophytic fungi, to compare the quantity of agarwood formation and to test the quality of agarwood formed through fungal infection.

Materials and Methods

Collection of Plant Samples

The plant parts of 10-year-old *Aquilaria malaccensis* Lam. were collected from agarwood plantation situated at Sin Gaung Lay village, Yat Kwet Gyi 13, Pyin Oo Lwin Township, Mandalay Region.

Isolation of Endophytic Fungi

The healthy plant parts such as leaves, bark, wood and fruit of *Aquilaria malaccensis* Lam. were used for the isolation of endophytic fungi. The isolation of fungi was done by the methods of Cui *et al*. (2011). The potato dextrose agar (PDA) medium and water glucose agar (WGA) medium were used for the macroscopical and microscopical characters of the fungi. The isolated fungi were identified by referring to Gilman (1957).

Analysis of Enzymatic Activities of Endophytic Fungi

The isolated fungal strains were screened for enzymatic activities such as cellulase, laccase, lipase, protease and pectinase by the methods of Sunitha *et al*. (2013), Bisht *et al*. (2017), Sharma *et al*. (2015) and Elsababty *et al* (2015).

Inoculation of Endophytic Fungi into *Aquilaria malaccensis* Lam.

The three strains of endophytic fungi such as *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and *Aspergillus* sp. (CM 11) were used as inoculation materials to inoculate *Aquilaria malaccensis* Lam. *Aspergillus* sp. (CM 2) were cultured in Czapek dextrose broth (CZB) and *Curvularia* sp. (CM 6), *Aspergillus* sp. (CM 11) were cultured in Potato dextrose broth (PDB). These fungal strains were incubated at 25˚C for 7 days at Microbiology Laboratory of Botany Department, University of Mandalay. Inoculation of endophytic fungi was carried out in an agarwood plantation situated at Sin Gaung Lay village, Yat Kwet Gyi 13, Pyin Oo Lwin Township, Mandalay Region from March 2024 to October 2024. The 13-years old *Aquilaria malaccensis* Lam. trees used in this study were randomly selected based on diameter at breast height (DBH) between 30 cm and 50 cm with height range between 5 m and 6 m. The fungal inoculation was done in *Aquilaria malaccensis* Lam. using syringe method. The holes were made along the trunks with an electric drill. The hole diameter was 0.5 cm and the inward depth was one-third of the stem diameter. The initial hole was made 30 cm above the ground. The vertical space between holes was 20 cm and in each horizontal line distributed three holes (Wahyuni & Prihantini 2020). 30 ml of homogenized fungal cultures was inoculated in each hole using a sterile syringe. A sterilized PDB medium was used as a control (Figure 1). The inoculation holes were subsequently covered with silicon to avoid contamination from insects and water (rain). Each treatment was taken three replications on different trees. The second times of fungal inoculation was carried out after 2 months of the first inoculation times.

Figure 1 Inoculation process of selected endophytic fungi into the stem of *Aquilaria malaccensis* Lam.

Collection of Induced Agarwood by Inoculating Endophytic Fungi

The induced agarwood was collected when the fungus inoculated on the stem of *Aquilaria malaccensis* Lam. trees. had grown for 3 months. The inoculation site changed in color from white to brown and was dug up by a chisel. The inoculated trees were cut down and inoculated trunks were weighed. The inoculated trunks were then carved to obtain the agarwood. The produced agarwood was collected and weighed (Wahyuni & Prihantini 2020). The yield of agarwood production was calculated using the following formula:

$$Y = \frac{\text{Output}}{\text{Input}} \times 100\ \%$$

Y = yield of agarwood production (%)

Input = log of wood before carving (kg)

Output = agarwood production after carving (kg)

Analysis of the Chemical Constituents in Essential Oils Agarwood (CM 11) of *Aquilaria malaccensis* Lam.

The agarwood induced by *Aspergillus* sp. (CM 11) was selected to extract the essential oil. The 50 g of agarwood powder was soaked in distilled water (800 ml) for 24 h. Hydrodistillation process was taken at 100 ˚C for 4 h to extract the oil (Liu *et al*. 2013). The chemical constituents in essential oils were analyzed by gas chromatography-mass spectrometry, GC-MS-QP2010-Ultra Shimadzu, at the Department of Chemistry, University of Mandalay.

Results

Morphological Characters of Endophytic Fungi

The macroscopical and microscopical characters of endophytic fungi isolated from the healthy plant parts of *Aquilaria malaccensis* Lam. were studied. The fourteen strains of endophytic fungi were tentatively designated as CM 1 to CM 14. The characteristics of selected endophytic fungi such as *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and *Aspergillus* sp. (CM 11) were described in Table 1 and Figure 2 - 4.

Enzymatic Activities of Endophytic Fungi

The enzymatic activities such as cellulase, laccase, lipase, protease and pectinase of the fourteen isolated fungal strains were tested. Among the fourteen fungal strains, the three fungal strains such as *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and *Aspergillus* sp. (CM 11) were showed the enzymatic activities (Table 2 and Figure 5 – 8).

Effect of Endophytic Fungi on Agarwood Production

In the present study, the stem of *Aquilaria malaccensis* Lam. was inoculated with selected endophytic fungi such as *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6), *Aspergillus* sp. (CM 11)

and Potato dextrose broth (Control) for inducing agarwood formation. The induced agarwood was harvested after 3 months of inoculation. The cross sections of the trees treated with selected endophytic fungi were showed in Figure 9. The average percentage of agarwood yield per tree treated with different fungal strains was estimated. The inoculation of *Aspergillus* sp. (CM 11) showed better results in average agarwood yield (3.5 %) per tree. The average agarwood yield per tree treated with *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and Potato dextrose broth (Control) was showed 1.9 %, 0.7 % and 0.2 % respectively (Table 3 and Figure 10). The chemical constituents in essential oils were analyzed by gas chromatography-mass spectrometry (Figure 11). The total 30 chemical compounds were identified, in which agarospirol (2.53 %), 2-Butanone, 4-phenyl- also known as benzylacetone (3.89 %), 2-Butanone, 4-(4-methoxyphenyl)- also known as anisylacetone (2.13 %), guaiol (2.74 %) and (-)-aristolene (2.41 %) are in the group of sesquiterpenes which is responsible for fragrance in agarwood (Table 4).

Table 1 Characteristics of Isolated Endophytic Fungi

Endophytic Fungi	Source	Macroscopical Characters		Microscopical Characters
		Surface	Reverse	
Aspergillus sp. (CM 2)	Leaf	White to black	White to cream	Conidiophores nonseptate, vesicle globose, phialide biseriate, conidia globose, brown
Curvularia sp. (CM 6)	Bark	Grey	Pale brown to black	Conidiophores brown, septate, conidia pale brown, ellipsoidal, 3-septate
Aspergillus sp. (CM 11)	Fruit	Olivaceous to brown	Pale yellow to olivaceous buff	Conidiophores nonseptate, vesicle globose, phialides uniseriate, conidia globose, hyaline

Table 2 Enzymatic Activity of Endophytic Fungi

Endophytic fungi	Cellulase	Laccase	Lipase	Protease	Pectinase
Aspergillus sp. (CM 2)	+	-	+	-	+
Curvularia sp. (CM 6)	-	+	-	-	-
Aspergillus sp. (CM 11)	+	-	+	-	+

+ = Positive reaction, - = Negative reaction

Table 3 The Average Yields of Agarwood Production after 3 months

Treatment	Wood (kg)	Agarwood (kg)	Yield (%)
Potato Dextrose Broth (Control)	19.21	0.043	0.2
Aspergillus sp. (CM 2)	11.16	**0.21**	**1.9**
Curvularia sp. (CM 6)	21.54	0.16	0.7
Aspergillus sp. (CM 11)	17.00	**0.60**	**3.5**

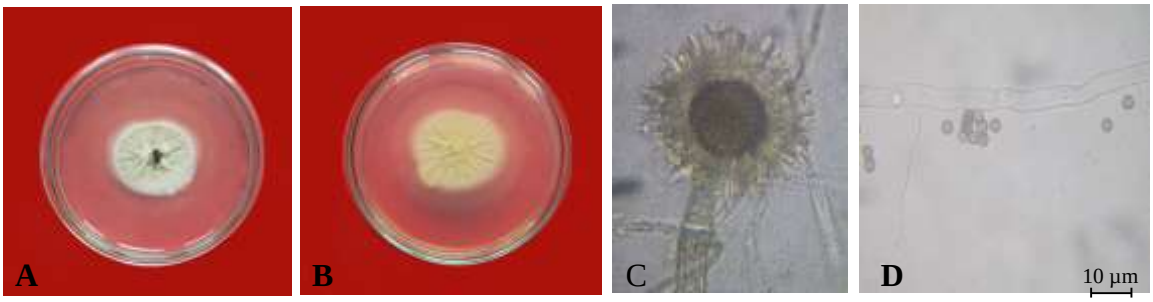

Figure 2 Macroscopical and microscopical characters of isolated *Aspergillus* sp. (CM 2) from leaves of *Aquilaria malaccensis* Lam. A. Surface colony (3 days), B. Reverse colony (3 days), C. Photomicrograph of a conidial head, D. Photomicrograph of conidia

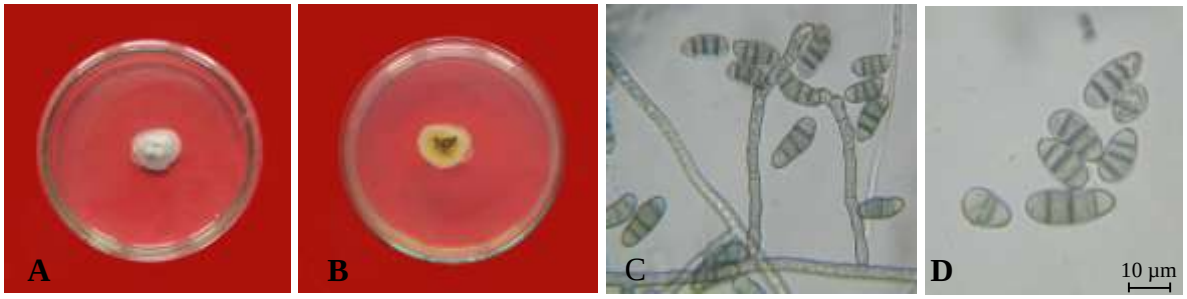

Figure 3 Macroscopical and microscopical characters of isolated *Curvularia* sp. (CM 6) from bark of *Aquilaria malaccensis* Lam. A. Surface colony (3 days), B. Reverse colony (3 days), C. Photomicrograph of conidiophore and conidia, D. Photomicrograph of conidia

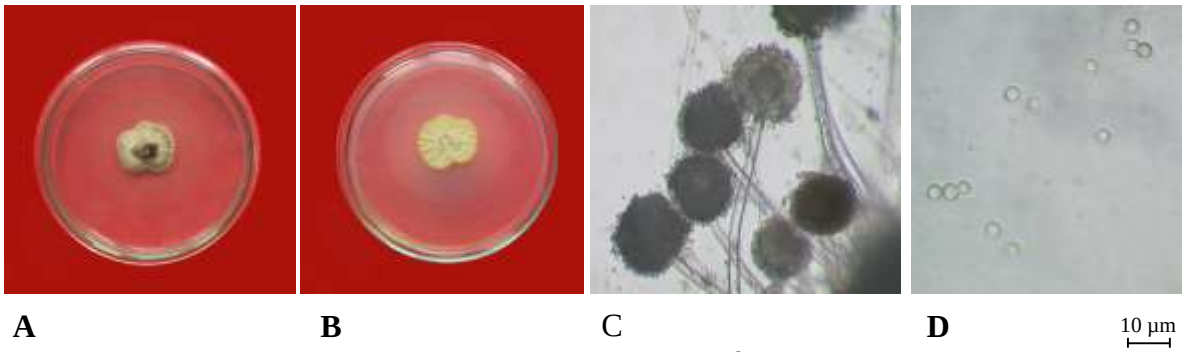

Figure 4 Macroscopical and microscopical characters of isolated *Aspergillus* sp. (CM 11) from fruit of *Aquilaria malaccensis* Lam. A. Surface colony (3 days), B. Reverse colony (3 days), C. Photomicrograph of conidial heads, D. Photomicrograph of conidia

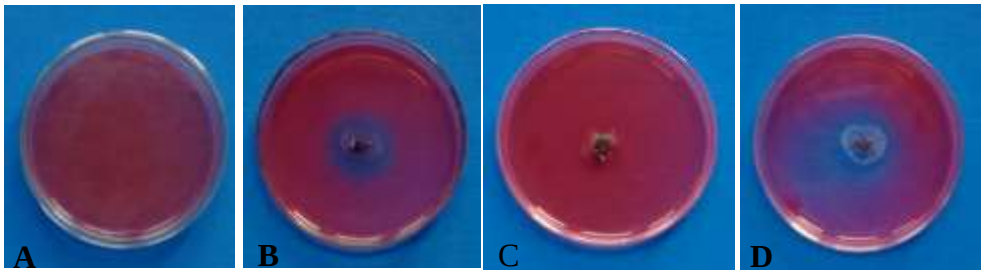

Figure 5 Cellulase Activities of Isolated Endophytic Fungi A. Control B. *Aspergillus* sp. (Positive) C. *Curvularia* sp. (Negative) D. *Aspergillus* sp. (Positive)

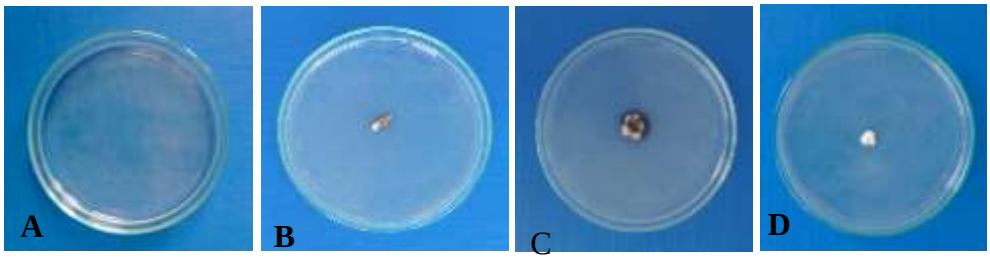

Figure 6 Laccase Activities of Isolated Endophytic Fungi A. Control, B. *Aspergillus* sp. (Negative), C. *Curvularia* sp. (Positive), D. *Aspergillus* sp. (Negative)

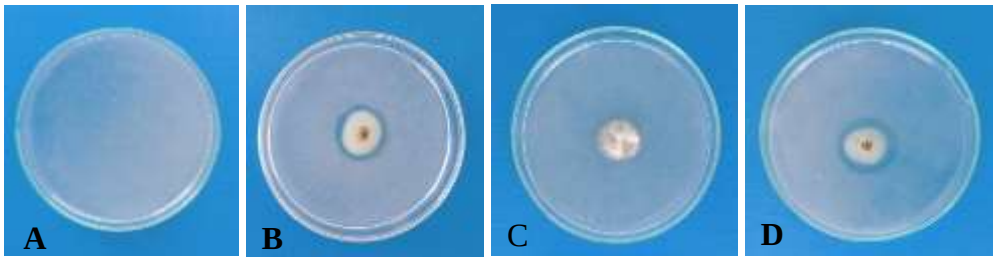

Figure 7 Lipase Activities of Isolated Endophytic Fungi A. Control, B. *Aspergillus* sp. (Positive), C. *Curvularia* sp. (Negative), D. *Aspergillus* sp. (Positive)

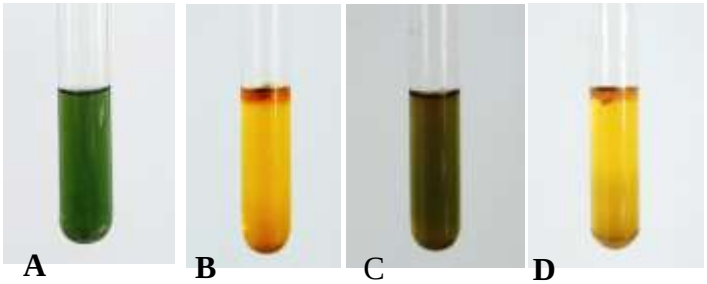

Figure 8 Pectinase Activities of Isolated Endophytic Fungi A. Control, B. *Aspergillus* sp. (Positive), C. *Curvularia* sp. (Negative), D. *Aspergillus* sp. (Positive)

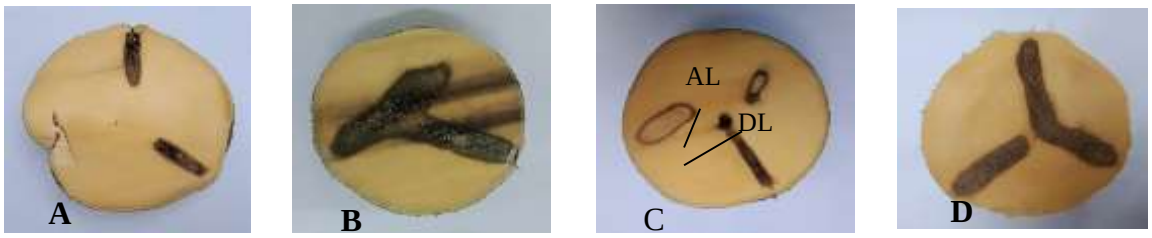

Figure 9 Agarwood formation in the stem of *Aquilaria malaccensis* Lam. after 3-monthtreatment with selected endophytic fungi A. Potato Dextrose Broth (control) B. *Aspergillus* sp. (CM 2) C. *Curvularia* sp. (CM 6) D. *Aspergillus* sp. (CM 11)

DL = Decaying layer, AL = Agarwood layer

Figure 10 Agarwood formation after 3-month treatment with selected endophytic fungi

A. Potato Dextrose Broth (control) B. *Aspergillus* sp. (CM 2)

C. *Curvularia* sp. (CM 6) D. *Aspergillus* sp. (CM 11)

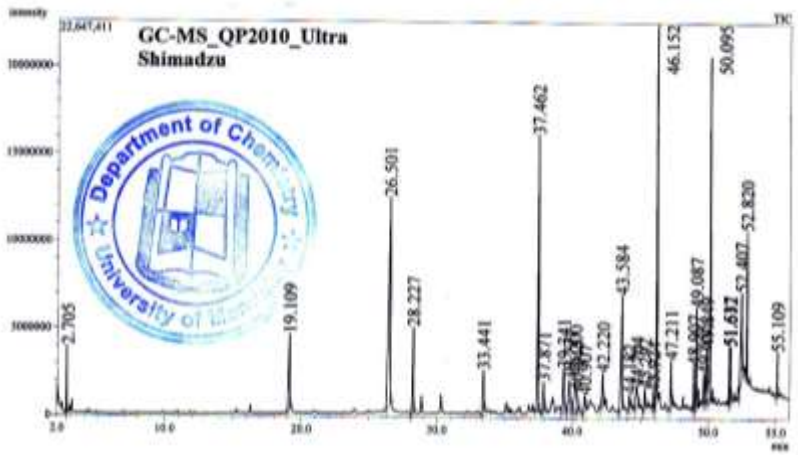

Figure 11 Chromatogram for GC-MS analysis of essential oils from agarwood (CM 11) of *Aquilaria malaccensis* Lam.

Table 4 Chemical composition of essential oils from agarwood (CM 11) of *Aquilaria malaccensis* Lam.

Peak#	R.Time	Area %	Name
2	19.109	3.89	2-Butanone, 4-phenyl-
5	33.441	2.13	2-Butanone, 4-(4-methoxyphenyl)-
8	39.341	2.53	Agarospirol
9	39.771	2.41	(-)-Aristolene
10	40.200	2.74	Guaiol

Discussion

In the present study, the fourteen endophytic fungi were isolated from healthy plant parts such as leaves, bark, wood and fruit of *Aquilaria malaccensis* Lam. The microscopical characters of CM 2 showed that conidiophores were nonseptate, vesicle globose, phialides biseriate, conidia globose, brown, one-celled. The microscopical characters of CM 11 showed that conidiophores were nonseptate, vesicle globose, phialides uniseriate, conidia globose, hyaline, one-celled. Gilman (1957) stated that the conidiophores of genus *Aspergillus* were nonseptate or septate, usually enlarging upward and broadening into elliptical, hemispherical or globose versicles bearing phialides; phialides uniseriate or biseriate; conidia spherical or elliptical, one celled. These characters of CM 2 and CM 11 were in agreement with the statements of Gilman (1957). Therefore, CM 2 and CM 11 were confirmed as *Aspergillus* sp.

The macroscopical characters of CM 6 colony were was grey. Reverse colony was pale brown to black in color. Conidiophores were brown, septate. Conidia were pale brown, ellipsoidal, 3-septate. Gilman (1957) stated that conidiophores of genus *Curvularia* were brown, septate, unbranched, conidia long-ellipsoidal, weakly curved, very pale brown, with three cross walls. These characters of strain CM 6 were in agreement with the statements of Gilman (1957). Therefore, CM 6 fungal strain was confirmed as *Curvularia* sp.

Abe *et al*. (2015) reported that the best genera for cellulase production were *Cladosporium, Aspergillus* and *Penicillium.* Sunitha *et al.* (2013) reported that the endophytic fungi *Aspergillus* sp. (Ci 10) and *Pestalotiopsis* sp. (Ci 4) isolated from *Calophyllum inophyllum* produced extracellular amylase, pectinase and proteinase. Fareed *et al.* (2017) reported that *Aspergillus calidoustus* fungi show the highest amount of lipase enzyme and *Penicillium marneffei* and *Aspergillus fumigatus* have no production of enzyme. Patel & Bhaskaran (2016) revealed that *Curvularia lunata* showed highest laccase activities (166.2 U/ml) on 3rd day of incubation in submerged medium.

In the present study, the fourteen isolated fungal strains were tested the enzymatic activities such as cellulase, laccase, lipase, protease and pectinase. *Aspergillus* sp. (CM 2 and CM 11) showed positive for cellulase, lipase and pectinase activities. These findings were agreed with Sunitha *et al.* (2013), Abe *et al*. (2015) and Fareed *et al*. (2017).

Curvularia sp. (CM 6) showed laccase activity. These findings were agreed with the statement of Patel & Bhaskaran (2016). All of the isolated endophytic fungi in this study did not show protease activity.

Wahyuni & Prihantini (2020) reported that the averages yield of production agarwood by simpori and implant techniques after six months of inoculation were 0.66 % and 0.64 % respectively. Subasinghe *et al.* (2022) studied agarwood resin inducement method using mycotoxins extracts of selected fungal species such as *Aspergillus niger* and *Fusarium solani* in *Aquilaria crassna*. The average resin contents of the individual trees inoculated with mycotoxins of the *Aspergillus niger* after seven months varied from 0.89 ± 0.35 % to 3.24 ± 0.86 % for a volume of 25 ml, from 0.89 ± 0.36% to 4.44 ± 1.50% for 50 ml and from 1.91 ± 0.10% to 2.20 ± 0.46% for 100 ml.

Among the fourteen fungal strains, the three strains of endophytic fungi isolated from healthy plant parts were used to assess their potential for induction of artificial infection in *Aquilaria malaccensis* Lam. *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6), *Aspergillus* sp. (CM 11) were isolated from healthy plant parts such as leaves, bark, fruit of *Aquilaria malaccensis* Lam.

The stem of *Aquilaria malaccensis* Lam. was inoculated with selected endophytic fungi and Potato dextrose broth (Control) for inducing agarwood formation. *Aspergillus* sp. (CM 11) after 3 months of inoculation showed better results in average agarwood yield (3.5 %) per tree. The average agarwood yield per tree treated with *Aspergillus* sp. (CM 2), *Curvularia* sp. (CM 6) and Potato dextrose broth (Control) was showed 1.9 %, 0.7 % and 0.2 % respectively. These results were agreed with the statements of Wahyuni & Prihantini (2020) and Subasinghe *et al.* (2022).

Agarospirol is one of the major responsible compounds for fragrance in agarwood. The presence of agarospirol compound in the agarwood confirmed the initiation of infection in the tree. The highest agarospirol content was measured in wood infected by fungi *Penicillium polonicum* (3.33 %) within 3 months (Chhipa & Kaushik 2017). Ahmaed & Kulkarni (2017) stated that agarospirol, benzylacetone, anisylacetone, guaiol and aristolene included in essential oil of *Aquilaria malaccensis* Lam.

In the present study, 2.53 % of agarospirol production was induced successfully using the fungi *Aspergillus* sp. (CM 11) within 3 months only, after inducing the infection. The other chemical constituents in essential oil included 2-Butanone, 4-phenyl- also known as benzylacetone (3.89 %), 2-Butanone, 4-(4-methoxyphenyl)- also known as anisylacetone (2.13 %), guaiol (2.74 %) and (-)-aristolene (2.41 %). These are in the group of sesquiterpenes which is responsible for fragrance in agarwood. These results were agreed with the statements of Chhipa & Kaushik (2017) and Ahmaed & Kulkarni (2017).

Endophyte infection may change the plant hormones, which can induce the plant defensive enzymes and stimulate production of plant secondary metabolites or even new compounds (Yang *et al.* 2022). The series of enzymes produced by endophytic fungi could play a major role in defense mechanism which ultimately becomes responsible for agarwood production.

In the present study, the agarwood resin formation can be induced in *Aquilaria malaccensis* Lam. by using selected endophytic fungi. Among the three fungal strains, *Aspergillus* sp. (CM 2 and CM 11) was indicated promising results (210 g and 600 g) in agarwood production within 3 months of infection. *Aspergillus* genus was confirmed to be the high potential agent for artificial production of agarwood.

Conclusion

The artificial induction of agarwood by means of fungal inoculation in this study has been proven to be a promising technique for agarwood formation. Therefore, *Aspergillus* sp. (CM 11) is an efficient and promising fungal strain for artificial agarwood producing during commercial-scale cultivation of *Aquilaria malaccensis* Lam. which can lead to the ecology and ecological stabilities for communities in near future.

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