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Effectiveness of Agarwood Formation Using a Fungal Consortium on Three Stem Diameter Sizes of *Aquilaria malaccensis* Lamk. in Banyuwangi

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ABSTRACT

Agarwood is a wood containing fragrant resin produced as a response of tree defense against disease. One of the artificial techniques that can be used for agarwood induction is inoculation, which is a technique of inserting pathogens (inoculants) into the stem such as fungi as a disease to accelerate the stimulation of agarwood formation. This study aims to analyze the size of the stem diameter against the length of infection propagation, the thickness of aromatic resin propagation, resin color and anatomical characteristics of agarwood to see the distribution of resin in the tissue, after being induced by a fungal consortium. Induction in this study was carried out on 3 sizes of tree trunk diameters (10 cm, 15 cm, and 20 cm) with 3 repetitions. Data were analyzed using analysis of variance (ANOVA) and qualitative descriptive for resin anatomy and color. The longest propagation of aromatic resin was obtained on a stem with a diameter of 15 cm. The aromatic resin that produced the darkest color was obtained from a stem with a diameter of 10 cm. The longest infection propagation was obtained on a stem with a diameter of 20 cm.The distribution of aromatic resin in the tissue accumulates in the pith rays, interxilary phloem, trachea, and tracheids.

Keywords: *Agarwood; inoculation; fungal consortium; Aquilaria malaccensis; Banyuwangi*

INTRODUCTION

Agarwood is a wood containing aromatic resin produced by various species of agarwood-producing trees from the Thymelaeacea family (Karlinasari et al., 2021; Kuspradini et al., 2016). Its high economic value and diverse benefits make this plant highly potential for development. The current price of class 1 natural agarwood is in the range of USD 20,000–100,000 per kg. Meanwhile, artificial agarwood ranges from USD 15,000–70,000 (Tin, 2023). Currently, agarwood has been widely used in the aromatic, medicinal, and religious fields (Naziz et al., 2019).

One tree species known to produce high-quality agarwood is *A. malaccensis* (Xie et al., 2024). Agarwood formation in nature occurs only by chance and is a very slow process (Kristanti et al., 2018). Agarwood only forms when the tree experiences wounds caused by various things such as animal scratching, insect borer attacks, branch breaks, lightning, and others. These wounds become entry points for infections such as microorganisms and chemicals, which can disrupt cell activity. In response to infection, the tree produces aromatic resin that helps suppress or prevent the infection from spreading (Akter et al., 2013; Mohamed et al., 2010; Turjaman et al., 2016; Zhang et al., 2014; Mulyaningsih, & Sumarjan, 2002).

Currently, artificial induction methods have been widely promoted to accelerate the stimulation of agarwood formation, one of which is the inoculation method, which uses biological agents (fungi) as inducers (Chen & Rao, 2022; Tabata et al., 2003). The principle of this method is to inject pathogenic fungi (inoculants) into the agarwood tree trunk to stimulate aromatic resin formation (Mulyaningsih, et al., 2014; Wangiyana et al., 2020). Several studies have confirmed the positive role of

fungi in agarwood induction, including *Fusarium solani, F. lateritium, Fusarium* sp. *Rigidoporus vinctus, Xylaris* sp., *Aspergilus, Lasidioplodia theobromae Penicilium polonicum, Arthtrinium, Syncephalastrum racemosum, Trichoderma asperellum, Rhizopus oryzae, Cunninghamela, Curvularia, Acremonium, Alternaria alternata, Cladosporium, Nigrospora sphaerica,* adn *Mucor* (Chen et al., 2018; Chhipa & Kaushik, 2017; Faizal et al., 2020; Lisdayani et al., 2015; Mohamed et al., 2010; Rachmawaty et al., 2021; Ramli et al., 2022; Tabata et al., 2003).

Fungal application to trees can be done in the form of a single inoculant or a mixture (consortium). Wulandar (2009) conducted inoculation with a consortium using *Acremonium* sp. and *Fusarium* sp. The results were that inoculation with a consortium tended to produce higher quality compared to a single inoculant seen from the intensity of wood color, the width of the color change zone and the level of aroma. Justin et al. (2020) also inoculated agarwood with 3 consortia from different fungal groups, namely *Trich oderma* with *Aspergilluss* sp., *Fusarium* with *Penisilium* sp., and *Trichoderma harzianum*, *Lasiodiplodia* sp. with *Curvularia* sp. The results were that all three consortia could induce agarwood.

Although several studies have shown consortia to be effective in inducing agarwood growth, each type of inoculant has varying strengths in inducing agarwood growth. This results in uneven colony infection at each inoculation hole. The level of fungal accumulation on the trunk can affect the speed of infection (Try et al., 2017). Fungal accumulation is closely related to the area of the trunk where the fungus accumulates. This study aims to analyze the effectiveness of tree trunk diameter on the length of infection propagation, mastic thickness, mastic color, and anatomical characteristics of agarwood, after induction with a fungal consortium.

METHODS

This research was conducted in two locations: first, the injection and sampling site in Pengatigan Village, Rogodjampi District, Banyuwangi Regency, for 2 months (October – December 2023); second, the anatomical observation site at the Advanced Biology Laboratory, Agarwood Study Center, Faculty of Mathematics and Natural Sciences, University of Mataram (August – September 2024).

Figure 1. Map of injection and sampling locations of *Aquilaria malaccensis* trees in Banyuwangi

The tools used in this study were an 8 mm diameter electric drill, a syringe (injection tool), straws, scissors, staples, labels, an axe/machete, a length measuring tool, a cell phone camera, and a microscope. The materials used in this study were 9 agarwood trees of the *A. malaccensis* species and a fungal consortium in liquid form containing 11 species of isolates that had been isolated from various regions.

The study was conducted using a Completely Randomized Design (CRD) with 3 treatments, namely induction treatment with a fungal consortium on stems with a diameter of 10 cm, 15 cm and 20 cm. Each treatment consisted of 3 replications, resulting in 9 experimental units. The induction process was carried out by drilling holes in the tree using an 8 mm diameter drill with a depth reaching 1/3 of the stem diameter and a drill angle of ± 45 degrees downwards. The holes were made in a circular pattern with a distance between holes of 10 cm horizontally and 20 cm vertically. Then, each hole that had been made was fitted with a straw as an aeration aid to flow the inoculant into the hole. Next, 8 ml of the fungal consortium was injected into each hole.

Observations were conducted two months after inoculation. Observations were made by peeling the bark around the inoculation hole to observe color changes. Then, the length of the infection path and the thickness of the mastic path were measured. Several wood samples from each treatment were then taken to observe their anatomical characteristics.

Agarwood anatomy was observed using a Zeiss Primo Star microscope with magnifications of 40 µm, 100 µm, and 400 µm. Anatomical observations were made to observe the presence of resin in the cells. Fresh agarwood preparations were made using the hand-free section slicing method. Agarwood specimens were cut to a length of 1 cm, after which they were fixed with 70% alcohol until the water bubbles in the wood disappeared. Slicing was done transversely and longitudinally. Next, the specimens were stained with Sudan III, then rinsed and covered with distilled water, then observed and photographed.

Data for the thickness of the propagation of mastic and the spread of infection were statistically processed using one-way analysis of variance (ANOVA) at a significance level of α= 5%. If significant results were found, further testing of the Least Significant Difference (LSD) was conducted. The color of mastic and the anatomy of agarwood were analyzed descriptively and qualitatively.

RESULTS AND DISCUSSION

Based on the results obtained in this study, the propagation of the mastic spread vertically and horizontally from the inoculation hole. The propagation of the mastic is characterized by a brown, linear color (**Figure 2**), which is produced as a defense response to infection (Suharti et al., 2011).

The vertical growth of mastic in **Table 1** shows that the highest growth was achieved on stems with a diameter of 15 cm, with an average value of 0.41 cm, significantly different from other treatments. These results indicate that a stem diameter of 15 cm is the best size for agarwood inoculation, considering the resulting growth thickness of mastic. Degradation of plant cell walls by pathogens triggers the biosynthesis of methyl jasmonate and salicylic acid, signaling molecules that activate plant defenses. During plant-pathogen interactions, these molecules rapidly accumulate at the infected site, triggering a hypersensitive response. This signal then spreads to other areas of the plant, inducing defense

responses such as the formation of phytoalexin compounds (Ramirez-Estrada et al., 2016).

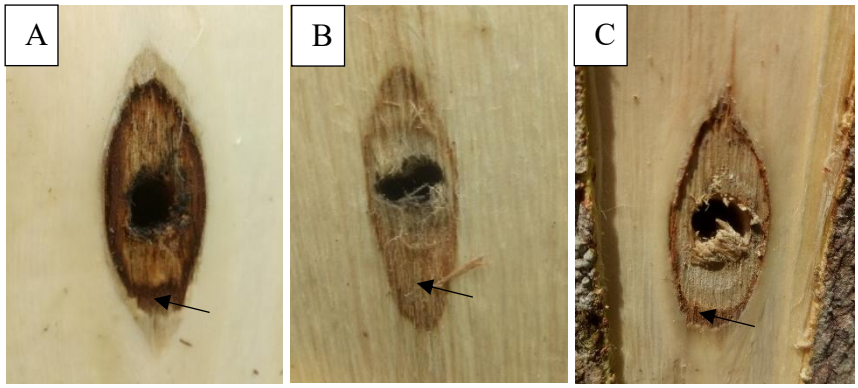

Figure 2. Thickness of the propagation of fragrant (↑) agarwood *Aquilaria malaccensis* after being induced by a fungal consortium, 2 months old. Description: A: Stem with a diameter of 10 cm; B: Stem with a diameter of 15 cm; C: Stem with a diameter of 20 cm.

Table 1. Thickness of mastic propagation on 3 sizes of *Aquilaria malaccensis* stem diameters after induction with a fungal consortium, 2 months old

No	Treatment	Length of propagation of fragrant resin (cm)			
		Vertical		Horizontal	
		Top	Below	Right	Left
1	P1: 10 cm	0,21 ± 0,17[a]	0,27 ± 0,24[a]	0,13 ± 0,05[a]	0,16 ± 0,07[a]
2	P2: 15 cm	0,41 ± 0,21[b]	0,78 ± 0,29[b]	0,14 ± 0,10[a]	0,18 ± 0,12[a]
3	P3: 20 cm	0,22 ± 0,09[a]	0,43 ± 0,36[a]	0,14 ± 0,07[a]	0,16 ± 0,07[a]

Notes: a, b, c and d are subsets of the BNT test; α = 0.05; means followed by the same letter/notation indicate no significant difference.

Li et al. (2022) stated that agarwood synthesis requires carbon from photosynthesis to be metabolized into agarwood. The carbon used in agarwood synthesis is stored in the form of non-structural carbohydrates (NSC), namely starch and sugar. Furthermore, Mulyaningsih et al. (2014) stated that agarwood production is influenced by the production of primary metabolites such as lignin, hemicellulose, total sugars, and total starch. The higher the production of primary metabolites, the higher the quality of the agarwood produced. According to Liu et al. (2022) agarwood begins to form from a series of physiological reactions in parenchymal cells where starch is converted into soluble sugars which then develop into resin, then transported and stored by the vessels to form agarwood.

A stem diameter of 10 cm produced the smallest thickness of mastic propagation and was not significantly different from the diameter of 20 cm. The resulting small mastic propagation is likely due to the smaller area of the stem diameter of 10 cm causing fungal invasion to be more limited so that fungal colonies are only concentrated in that area. This allows defense signals to take a shorter time to spread, recognize pathogen infection more quickly, but the resin production capacity is more limited. Defense signal molecules such as salicylic acid and jasmonic acid diffuse through the phloem sieve tubes and move to neighboring cells via plasmodesmata to activate defense responses such as sesquiterpene formation

in agarwood (Li et al., 2020; Rasool & Mohamed, 2016). According to Muffei dan Bossi (2006) Muffei and Bossi (2006), signal spread depends on time and distance.

Meanwhile, a 20 cm diameter tree is the largest, but it also has a low resin propagation rate. This indicates that although a 20 cm diameter tree has the highest infection rate, it does not necessarily mean that it produces a high amount of resin. Several possible reasons for the low resin propagation rate of a 20 cm diameter tree are possible. First, the larger diameter allows the fungus to more freely invade the tree, while the tree's defense response is likely slower due to its larger diameter, requiring more time for the plant's defense signals to propagate. This results in slower or even no resin production due to the wood's decay. Second, because the 20 cm diameter tree is larger, it likely has more old leaves than young leaves as a source of photosynthates. Injured tissue will absorb the resulting photosynthate to accumulate as a defense substance. The source tissue's ability to respond to stimuli to deliver photosynthate, which is used to form defense compounds such as terpenes, decreases with tissue age and the increase in leaf branches (Schultz et al., 2013).

The horizontal spread of mastic tends to be smaller than the vertical spread. This is because the vertical spread of infection is higher, resulting in the tree producing more mastic to suppress the spread of fungal infection.

Microscopic observations show that a 10 cm diameter stem produces the darkest color with dark brown resin clumps (**Figure 3**). A 15 cm diameter produces a yellowish resin. Meanwhile, a 20 cm diameter produces a reddish resin that is still in liquid form. The color of the resin indicates the thickness of the resin deposit. The thicker the resin deposit, the blacker the color, conversely, thin agarwood deposits appear like a yellowish to reddish liquid. Thin resin filling can crack after drying as seen in **Figure 4E** and **B**. These observations show that a 10 cm diameter stem has the best color, followed by a 15 cm diameter, then a 20 cm diameter.

The formation of mastic on trunks measuring 10 cm in diameter likely occurs earlier due to the tree's faster response to fighting pathogens. Therefore, the resulting mastic appears darker (**Figure 3A and B)**, and the resulting mastic deposits are thicker when viewed microscopically. According to Rasool dan Muhammad (Rasool & Mohamed, 2016) after pathogen infection, color changes will be visible around the wound within a few days. This color darkens over time and is easily detected against white wood. Selno et al. (2021) stated that the darker the agarwood color, the more mastic content has accumulated in the wood tissue.

The filling of fragrant resin in the stem of *A. malaccensis* agarwood after 2 months of inoculation filled the pith ray cells, interxilary phloem, trachea and tracheids as seen in **Figure 3**. The accumulation of fragrant resin in the tissue did not occur evenly because the harvest time was shorter, namely 2 months. Filling of fragrant resin in the tracheids only occurred in the treatment of 20 cm stem diameter (**Figure 3C and F**). The initial filling of resin occurred in the pith ray and interxilary phloem (Mulyaningsih et al., 2014). The tree will block or plug these cells with resin because the fungus will spread to these areas (Cui et al., 2013).

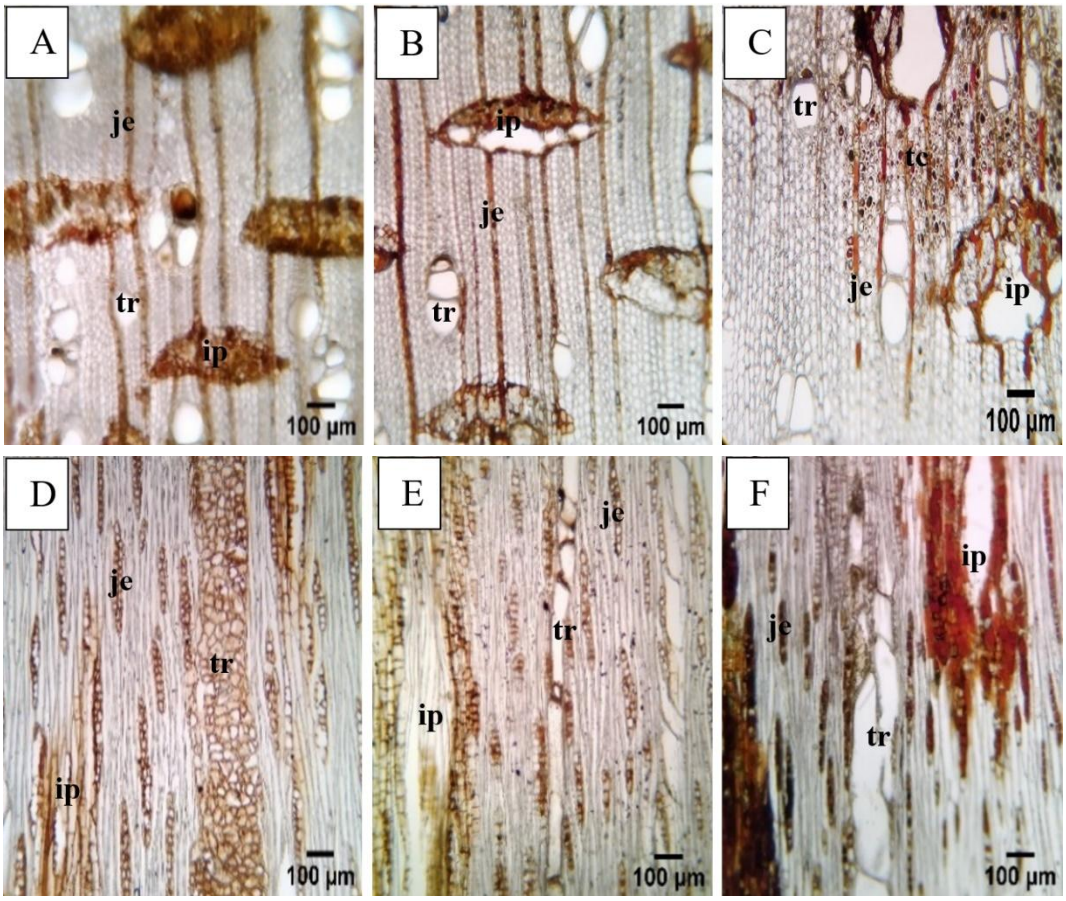

Figure 3. Cross-section (A – C) and longitudinal (D – F) of *Aquilaria malaccensis* agarwood after induction with fungal consortium, 2 months old. Description: A and D: stems with a diameter of 10 cm; B and E: stems with a diameter of 15 cm; C and F: stems with a diameter of 20 cm; je. Pith radius; ip. Interxilary phloem; tr. Trachea.

In this study, fungal hyphae in a 20 cm diameter stem were not only spread to cells containing food or starch, but also spread to tracheids (**Figure 1 D**). Therefore, the plant tries to block all cells near the infection by spreading resin to the area as a form of defense because the plant feels threatened (Prastyaningsih et al., 2015). Meanwhile, according to Liu et al. (2022) when there is excessive resin accumulation in the interxilary phloem and pith rays, the resin is transported and appears in the vessels and wood fibers adjacent to the parenchyma cells. The resin adheres to the vessel walls and diffuses through the nodes to adjacent vessels. The resin in the vessels diffuses axially to the upper and lower ends.

The filling of the mastic in the trachea and tracheids as seen in **Figure 4**, some are completely filled and some are only partially filled. According to Mulyaningsih et al. (2014), trachea and tracheids are storage areas for mastic, so if the mastic fills the tracheid and tracheal cells completely, the resulting agarwood will have a darker color. The mastic in the tracheal tissue accumulates in the tracheal walls (**Figure 4E**), tracheal sieves (**Figure 4G**), and the tips of the tracheal sieves (**Figure 4F**).

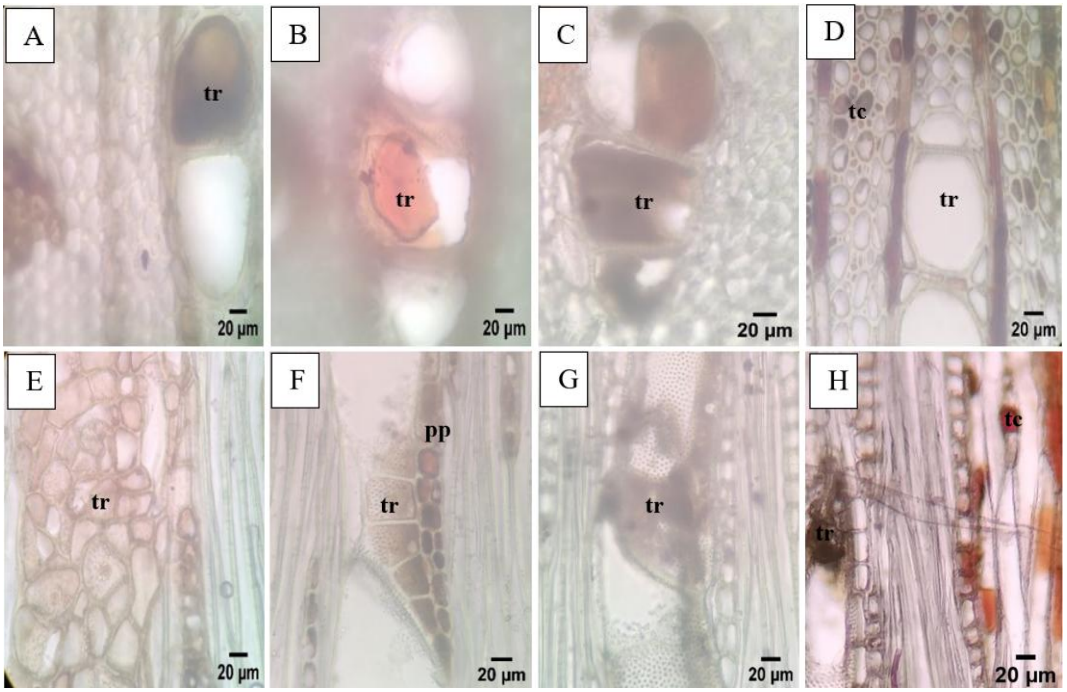

Figure 4 Filling of resin in the trachea and tracheids of *Aquilaria malaccensis* agarwood stems after induction with a fungal consortium, 2 months old. Description: A – F: Stems with a diameter of 10 cm; C and G: Stems with a diameter of 15 cm; C – H: Stems with a diameter of 20 cm; tr. Trachea filled with resin; tc. Tracheids filled with resin; pp. Paratracheal parenchyma filled with resin.

The observation results in **Table 2** show that the highest average value of the vertical upward spread of infection was obtained from the 20 cm stem diameter treatment, which was 1.37 cm. This result was significantly different from the 10 cm stem diameter treatment but not significantly different from the 15 cm stem diameter treatment. Meanwhile, the highest vertical downward spread of infection was obtained from the 15 cm stem diameter treatment, which was 2.1 cm and this result was significantly different from the 10 cm stem diameter treatment but not significantly different from the 15 cm stem diameter treatment. The difference in fungal infection spread at a 10 cm stem diameter is likely due to trees with a smaller diameter having a faster defense response so that the spread of fungal infection can be suppressed. Meanwhile, according to Jalil et al. (2022) a smaller tree diameter means having less xylem so that the spread of fungi to infect the tree trunk is more limited.

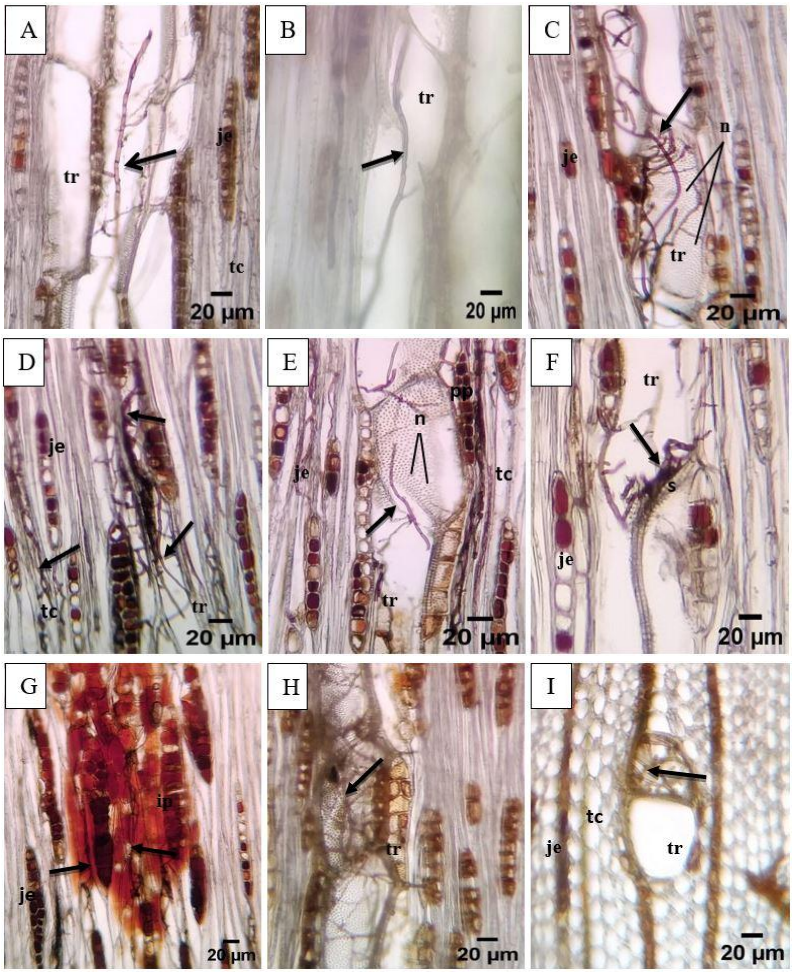

Figure 5 Longitudinal (A - H) and transverse (I) cross sections of the stem of *Aquilaria malaccensis*. Captions: A. Hyphae in the xylem with a stem diameter of 10 cm; B. Hyphae in the xylem with a stem diameter of 15 cm; C - I. Hyphae in the xylem with a stem diameter of 20 cm; arrow (↑): fungal hyphae; je. Pith rays; ip. Interxilary phloem; tr. Trachea; tc. Tracheids; pp. Paratracheal parenchyma; n. Nodules

The average length of downward vertical fungal infection propagation is higher when compared to upward vertical. This phenomenon is likely caused by the force of gravity, which causes fungal consortia to be absorbed more quickly downward. Fungal hyphae that infect tree trunks are septate (**Figure 5A–G**) and some are non-septate (**Figure 5H–I**). Fungi will occupy areas containing photosynthates such as pith rays (**Figure 5A**) and interxilary phloem (**Figure 5G**) as well as water transport networks such as trachea (**Figure 5 B, C, D, E, F, H, and I**) to grow and develop (Suharti et al., 2011). Fungi will degrade the phloem first and will form colonies after the wood is infected. These colonies then spread to other wood cells until biochemical reactions completely prevent their spread (Adams et al., 2016; Jalil et al., 2022). Aaccording to Liu et al. (2019) the fungus enters the tree and moves axially along the trachea (**Figure 5**) and radially along the pith radii to accumulate and induce agarwood production in the interxylary phloem and pith radii.

Table 2. Length of agarwood infection propagation on 3 sizes of *Aquilaria malaccensis* stem diameters after induction with fungal consortium.

No	Treatment	Length of infection propagation (cm)			
		Vertical		Horizontal	
		Top	Below	Right	Left
1	P1: 10 cm	0.94 ± 0.31^{a}	1.27 ± 0.43^{a}	0.32 ± 0.12^{a}	0.32 ± 0.09^{a}
2	P2: 15 cm	1.18 ± 0.17^{ab}	2.1 ± 0.28^{b}	0.33 ± 0.12^{a}	0.31 ± 0.17^{a}
3	P3: 20 cm	1.37 ± 0.26^{b}	1.94 ± 0.21^{b}	0.28 ± 0.09^{a}	0.36 ± 0.11^{a}

Notes: a, b, and c is a subset of the BNT test: $\alpha = 0.05$; means followed by the same letter/notation indicate no significant difference.

The horizontal spread of infection was not significantly different across all treatments. The resulting spread was very small because the fungus tends to follow the direction of the vascular tissue in the vertically arranged plant stems (Santoso, et al., 2007). The fungus's difficulty in penetrating wood cell walls, which are composed of cellulose, hemicellulose, pectin, and lignin, prevents it from invading in a wider horizontal direction (Lorrai & Ferrari, 2021). Zhao et al. (2024) reported no increase in horizontal spread during the first 3 months after inoculation. However, it continued to expand from the 3rd to the 6th month. Meanwhile, in this study, agarwood was harvested 2 months after inoculation.

CONCLUSIONS

The results of the study can be concluded that the longest propagation of mastic was obtained on a stem with a diameter of 15 cm, both upwards 0.45 cm and downwards 0.78 cm, significantly different from the other two treatments. However, the darkest color of mastic, namely dark brown, was obtained on a stem with a diameter of 10 cm. The longest infection propagation was obtained in the treatment of a stem diameter of 15 cm, downwards 2.1 cm, which was significantly different from a stem with a diameter of 10 cm. The spread of mastic in the wood tissue of *Aquilaria malaccensis* accumulated in the interxilary phloem cells, pith rays, trachea and tracheids.

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