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Full Length Research Article

Agarwood Formation in *Gyrinops versteegii* Seedling Stage using Four Types of Inducers

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ABSTRACT

Agarwood is decayed wood that contains aromatic resins. It is formed biotically by bacterial, fungal, and physical infections due to injuries, such as broken branches, insect and mammal attacks, lightning strikes, and fires. The natural formation of agarwood has encouraged the development of artificially induced agarwood through inducer inoculation. This study aimed to determine the formation of agarwood at the seedling stage, the quality of agarwood produced, and the best inducer to induce agarwood. The research was conducted in March-June 2021 at the Greenhouse of the Faculty of Mathematics and Natural Sciences, Mataram University, Lombok, West Nusa Tenggara. The research method used was a complete randomized design consisting of four inducer treatments such as distilled water (control), liquid inducer, gel inducer, and paste inducer. Each treatment was repeated three times. Twelve experimental units were obtained. Data were analyzed using an analysis of variance, followed by the least significant difference test if the results were significant. The results showed that inoculation of *G. versteegii* at the seedling level using four inducers could produce agarwood that is classified in *kamedangan* class. Inoculation using a gel inducer resulted in the highest *kamedangan* quantity, and the liquid inducer produced the longest transmission of 10.83 cm and the best *kamedangan* quality.

1. Introduction

Gaharu (*Gyrinops versteegii*) is a tree species of the *Gyrinops* genus that produce agarwood (Faizal et al. 2020; Iryadi et al. 2021; Sukito et al. 2020). Based on variations in morphology, wood anatomy, and phytochemicals, *G. versteegii* consists of five groups or provenances, including Beringin, Buaya, Madu, Pantai, and Soyun provenances (Mulyaningsih et al. 2014). Agarwood is wood parts that contain resin and have a fragrant aroma. The formation of agarwood starts from a wound in the wood that is infected by disease (Huang et al. 2016). The *G. versteegii* is well-known as an agarwood-producing tree in Lombok Island (Akter et al. 2013; Hou 1960; Mulyaningsih and Yamada 2008).

The plantation of *G. versteegii* trees in Lombok was started in the reform era due to the government programs that had expectations of the agarwood's high price (Al Hasan and Wahyuni 2019). According to the BSN (2011), agarwood was grouped into three classes: agar (*gubal*), dhum (*kamedangan*), and dust (*abu*). The *gubal* class is divided into five quality classes, namely double

super, super A, super B, medium-super (*super tanggung*) A, and medium-super (*super tanggung*) B. The *kamedangan* class is divided into six quality classes, namely *sabah*, *kamedangan* A, *kamedanga*n B, TG.C, green *kamedangan*, and white *kamedangan*. The dust class is divided into two grades, namely *gubal* dust and *kamedangan* dust. According to Womsiwor et al. (2018), the price of agarwood in Indonesia for the *gubal* super, *kamedangan sabah*, and *gubal* dust class reached IDR 200 million/kg, IDR 1.5 million/kg, and IDR 50,000/kg, respectively. Meanwhile, Septianingrum et al. (2015) stated that the average price of agarwood reached IDR 45.2 million/ton.

Gubal of *G. versteegii* can be used as one of the ingredients in the perfume, incense, cosmetic, medicinal, and antioxidant manufacturing industries (López-Sampson and Page 2018; Mulyaningsih 2021; Siran and Turjaman 2010; Try et al. 2017). *G. versteegii* leaf extract contains bioactive compounds in the form of alkaloids, phenols, flavonoids, saponins, tannins, and terpenoids which synergize as immunomodulators in the body so that *G. versteegii* leaves can be used as a tea (Sulistyani et al. 2016). Another use of the acetone extract of infected *G. versteegii* wood showed high antioxidant and antidiabetic activity due to phenolic and flavonoid compounds in the infected wood. The first reports of antidiabetic activity were found in the wood extract of *G. versteegii* from Lombok (Sukito et al. 2020).

The natural formation of agarwood requires a very long process, about ten years (Tan et al. 2019). Meanwhile, the company's demand for agarwood is very high at 5,238 tons per year (Azren et al. 2019; BPS 2015). The high demand for agarwood has encouraged the development of various artificial inoculation methods in which agarwood formation is artificially induced. The artificial formation can produce agarwood in a shorter time. However, the aroma of the resin produced is different from the resin produced naturally (Kadir et al. 2020). Therefore, an artificial induction method was developed to intensify agarwood production in terms of yield and quality. Local farmers use artificial induction with traditional methods such as nailing, perforating, burning, breaking stems, and removing bark. The development of artificial induction is increasingly interesting because it uses microorganisms as inducers. The microorganisms used are fungi; besides being attractive by artificial induction, they also have a higher demand from entrepreneurs and plantation owners (Azren et al. 2019). There are several fungal species often used in the induction of agarwood formation, including *Cunninghamella bainieri, Fusarium solani, Rigidoporus vinctus, Cercosporella* sp., *Chaetomium spiral, Cladosporium* sp., *Phialogeniculata* sp., *Pithomyces* sp., *Rhizopus* sp., *Spikulostibella* sp., *Trichoderma* sp., and *Lasiodiplodia theobromae* (Akter et al. 2013; Rasool and Mohamed 2016; Chen et al. 2018; Faizal et al. 2020).

Many studies on agarwood inoculation have been reported. For example, Mohamed et al. (2014) induced three-year-old *Aquilaria malaccensis* trees and could produce agarwood. The present study induced *G. versteegii* at the seedling stage using four types of inducers, which are aquadest and fungi inoculant in liquid, gel, and paste forms. The inducers were chosen because they are easy to be transported and inoculated into *G. versteegii*. In addition, a study on agarwood inoculation using these inducers has never been reported before. Therefore, this study aimed to determine the formation of agarwood at the seedling level, the quality of *gubal* produced, and the best inducer to induce agarwood.

2. Materials and Methods

This research was conducted at the Green House of the Faculty of Mathematics and Natural Sciences, Mataram University, Mataram, Lombok, West Nusa Tenggara, in March-June 2021 for 75 days. The ambient temperature in this study was between 27°C in the morning to 32°C in the afternoon. Meanwhile, the humidity reaches 80% in the morning to 60% in the afternoon. The average light intensity used in this study is 80% during the day.

2.1. Materials

The materials used in this study were 12 individual gaharu (*Gyrinops versteegii*) trees at the seedling level of 3-year-old, with an average seedling height of 100 cm and trunk diameter of 0.7 cm. The *G. versteegii* seedlings were obtained from Spakek Village, Pringgarata District, Central Lombok. Polybags with a diameter of 25 cm and a height of 25 cm were used as the planting media containers. Plastic grafting of 3 cm wide was used to cover the inoculation wound. The 80% paranet was used to keep the temperature low. Four types of inducers were used to induce the formation of agarwood in this study, namely aquadest as a negative control and fungi species belonging to the Ascomycetes class as an inducer in liquid, cream, and semi-gel forms to induce the formation of agarwood.

2.2. Methods

This research was conducted in nature and was performed by a completely randomized design with four treatments and three replications so that 12 experimental units were obtained. The first treatment of trees was induced using distilled water (negative control). The treatment of the two trees was induced by using a liquid form of a fungal inducer belonging to the Ascomycetes class. The treatment of the three trees was induced using a semi-gel form of fungal inducer species belonging to the Ascomycetes class. The treatment of the four trees was induced using a fungal inducer species belonging to the Ascomycetes class in the form of a paste. Each treatment was repeated three times. The inducer was inoculated as much as 0.5 mL in each tree wound.

The induction process on agarwood seedlings with an average height of 100 cm was carried out using two methods, namely using a 1.2 mm drill bit for perforating and a knife for stripping the skin. Perforation was carried out at the height of 20 cm, 30 cm, and 40 cm, and skin-stripping at the height of 50 cm, 60 cm, and 70 cm from the ground surface. Finally, the puncture wound was closed directly using grafting plastic, while the peeling skin was closed again by wrapping it with grafting plastic.

Observations were made at the age of 75 days after inoculation (DAI) by peeling the bark to observe changes in the color of the wood. Post-harvest handling is done by separating agarwood from healthy wood by scraping (crafting). Furthermore, it is sorted and selected between agarwood and agarwood dust (Mulyaningsih et al. 2014). The agarwood yields were further classified based on SNI 7631: 2011 (BSN 2011). The wet weight of the agarwood produced was then measured, followed by air-drying the agarwood at room temperature for three days and then weighed to determine its dry weight.

Twelve agarwood specimens were cut along 0.5 cm to make fresh preparations, then vacuumed until the specimens were submerged. Fresh agarwood was prepared using the standard freehand section (Lux et al. 2005; Berden 2020). The stem samples were sliced transversely and

longitudinally in the tangential section using a sharp (new) razor blade, resulting in a very thin incision. The incision is placed on a glass object that has been dripped with water. The water in the object-glass is absorbed using tissue paper until the object-glass is clean. The specimens were then dripped with distilled water as a covering medium, then observed using a digital microscope Zeiss Primo Star, Switzerland, with magnifications of 40 µm, 100 µm, and 400 µm and photographed.

The data observed were changes in stem color, agarwood transmission, agarwood in cells, wet and dry weight of agarwood, and a class of agarwood (Mulyaningsih et al. 2014). Observational data were analyzed using variance (ANOVA) to determine the effect of inducer type on agarwood formation. If the ANOVA results are significantly different, proceed with the Least Significant Difference (LSD) further test.

3. Results and Discussion

The results showed that the inducer inoculation could produce medium-grade agarwood (**Fig. 2–4**). The medium-grade agarwood is characterized by its white-cream to light brown color. In addition, the wood fiber cells filled with aromatic resin are not yet complete, the wood is soft, and the smoke from the combustion has a slightly fragrant (weak) aroma (BSN 2011). According to Samsuri (2013), the formation of good quality agarwood grows in areas with a temperature of 28-34°C and humidity of 60-80%. In addition, agarwood-producing trees in *A. malaccensis* can be induced on young trees aged three years and harvested after six months of inoculation, which can produce changes in the color intensity of the wood (Mohamed et al. 2014).

3.1. Post-Inoculation Physical Response

Shoots began to appear in the second week at 30 cm height between the first and second inoculation holes (**Fig. 1 and Fig. 2**). All treatment trees have grown new shoots from the third week to the fifth week to the fifth week. It indicated that the tree gives an excellent response to the treatment given. Observation of the sixth week of the stem of the former inoculation hole changes shape and enlarges (**Fig. 2c and Fig. 2g**). Meanwhile, in the stripping wound, the stem regenerates, and the wood looks a bit darker than the original (healthy) bright white wood. Dwianto (2019) explained that the characteristics of plant parts that have produced agarwood are that the bark becomes soft, the leaves of the plant turn yellow and fall off, and there is swelling or thickening of the stems and branches of the plant.

The physical response observed from each inoculated tree indicated that the agarwood resin was forming soon. This response results from physical injury so that the tree becomes weak and susceptible to fungal infection. The wood around the wound darkens and is easily detectable against the initially white wood (Rasool and Mohamed 2016). Symptoms of agarwood formation became more visible in the seventh week of observation. The torn bark showed dark wood color and the blackening of the bark around the inoculation site. Agarwood formation takes 2-3 months after inoculation (Liu et al. 2018). The initial formation of agarwood is marked by the blackening of the bark around the injured stem (Chong et al. 2015).

Fig. 1. The three years old seedlings of *G. versteegii* before inoculation.

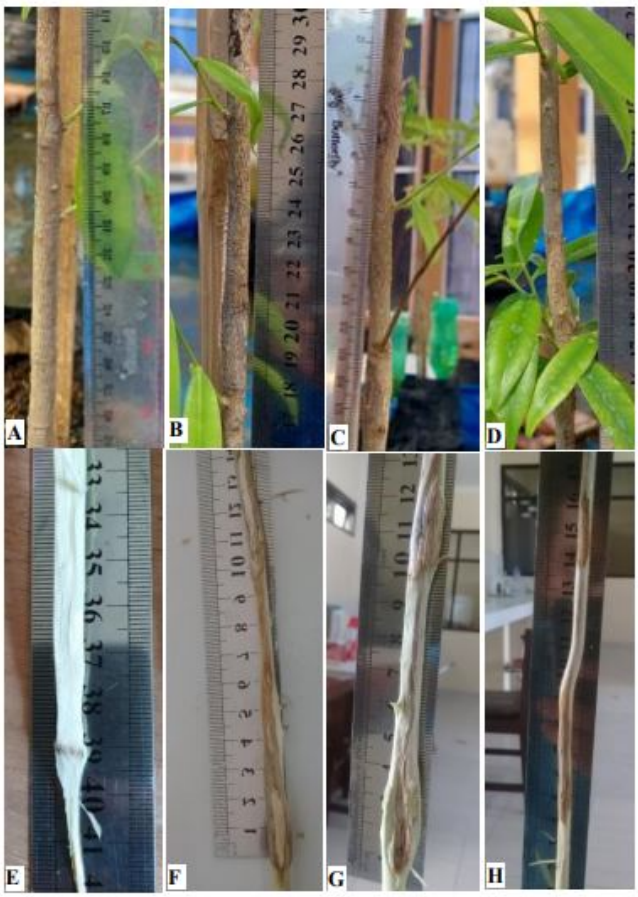

Fig. 2. Seedling stems of *G. versteegii* that have been inoculated with four different inducers aged 75 DAI (the day after inoculation): (a) negative control before stripping, (b) liquid inducer before peeling, (c) inducer gel before peeling, (d) inducer paste before peeling, (e) transmission of agarwood in the negative control, (f) transmission of agarwood in the liquid inducer, (g) transmission of agarwood for the gel inducer, and (h) transmission of agarwood on the paste inducer.

The induced agarwood seedlings responded with different time intervals based on the type of inducer given. For example, treatment with a liquid inducer caused the most rapid symptoms at 8 h after inoculation, indicated by wilted leaves along the stems of the holes. On the other hand,

symptoms observed in dry leaves indicate that the tree responds to the treatment given. Furthermore, the paste inducer also resulted in a response characterized by wilting and yellowing of the leaves 24 h after inoculation (**Fig. 1**). According to Triadiati et al. (2016), leaf symptoms are a response due to tree injury and infection from fungi. However, the gel inducer indicated a different response, namely the enlargement of the stem around the inoculation hole (**Figs. 2C and G**). According to Mohamed et al. (2014) and Try et al. (2017), stem enlargement is caused by excessive accumulation of fungi in stems, thus triggering the formation of caline hormones in the companion cells. The growth hormone formed will stimulate the wood cell's growth into the callus and eventually form a gall around the wound.

3.2. Agarwood Formation Process

Mature agarwood-producing *A. crassna* trees can withstand the toxic substances contained in the inducer material (Try et al. 2017). The higher infection caused by fungi will increase the resin yield produced as the host tree responds to fungal growth in the trunk (Akter et al. 2013). Infected wood produces agarwood of *kamedangan* class with characteristic wood color changing to light brown. Based on SNI 7631: 2011 (BSN 2011), agarwood of *kamedangan* class contains mastic with a weak aroma, characterized by its color, grayish-white to brownish-brown, coarse fibrous, and softwood.

The anatomy of the agarwood produced showed the presence of light brown to dark brown resin in the trachea cells, interxylary phloem, and pith radius (**Fig. 3**). The resin formed in the cell is due to the accumulated starch as the main ingredient for resin formation. Phloem interxylary cells and xylem pith radius are the site of resin formation at the beginning of agarwood formation (Liu et al. 2018; Mulyaningsih and Sumarjan 2002; Mulyaningsih, et al. 2007). The anatomy of the stem in the liquid inducer treatment had a better appearance quality, indicated by the resin that had undergone agglomeration and was dark brown in color (**Fig. 3e**). Unlike the gel and paste inducer treatment, the resin formed was still a liquid with a light brown color (**Figs. 3 and 4**). The liquid inside the cell adheres to the cell walls of the trachea and pith radius cells due to the adhesion force between the resin compound and the cell wall. Adhesion forces occur because of the attractive forces between dissimilar particles (Kesuma and Kasmungin 2015). In this study, the resin did not fill the cell space, as observed from the resin at the end of the cell (**Figs. 4D and E**). It indicated that resin formation is still ongoing at the time of harvest (Try et al. 2017).

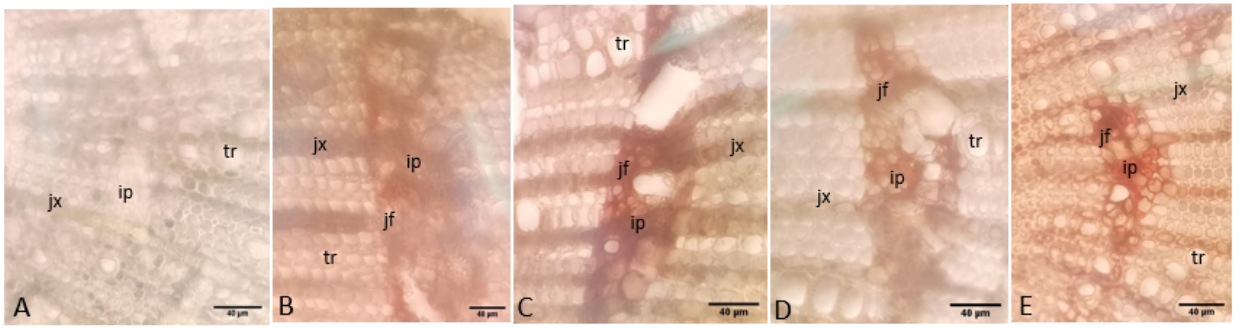

Fig. 3. Cross-section of the stem of a *G. versteegii* seedling inoculated with four different inducers aged 75 DAI: (a) healthy stems, (b) negative control, (c) liquid inducer, (d) gel inducer, (e) paste inducer, (f) ip = interxylary phloem, tr = trachea, jx = xylem pith radius, and jf = phloem pith fingers.

The resulting agarwood will be deposited first on the pith radius, interxylary phloem, and trachea (**Figs. 3 and 4**). Agarwood is formed starting from the tissue that transports the products of photosynthesis, such as the pith radius, interxylary phloem, to the wood parenchyma and the tracheal cells. The first agarwood is in the form of a liquid that accumulates more in the wood cells and is then deposited in the wood cells, the form of brown lumps, denser lumps, and darker color (Mulyaningsih and Sumarjan 2002; Tabata et al. 2003).

3.3. The Physiological Process of Agarwood Formation

Fungi that accumulate around the wound can inhibit the transport of carbohydrates from photosynthesis through the phloem, stimulating the plant to secrete defense compounds, namely sesquiterpenoids (Triadiati et al. 2016; Try et al. 2017). Taiz and Zeiger (2010) stated that sesquiterpenoids are plant defense compounds of the phytoalexin type. Sesquiterpene compounds are one of the secondary metabolites derived from the biosynthesis of *isopentyl* and *dimethylallyl pyrophosphate*. This secondary metabolism is produced through the mevalonate pathway and the *methylerythritol phosphate* (MEP) pathway and then produces *isopentenyl diphosphate* or *dimethylallyl diphosphate*, which can form sesquiterpene compounds.

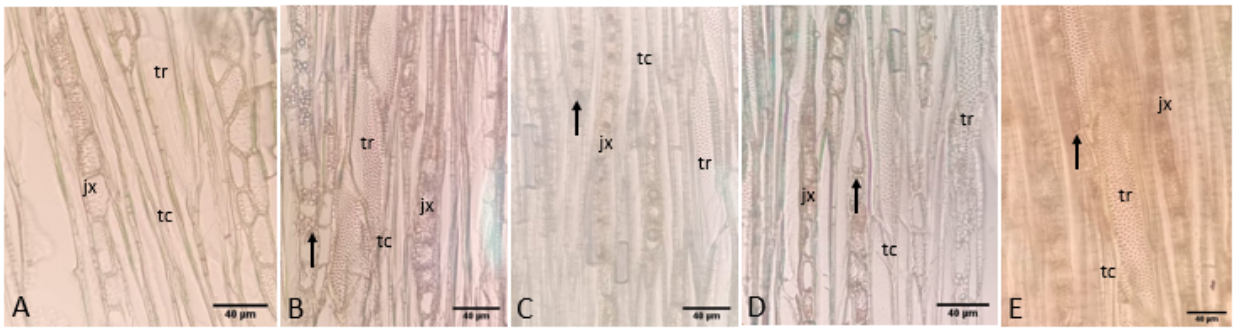

Fig. 4. Tangential cross-section of *G. versteegii* seedling stem inoculated with four different inducers aged 75 DAI: (a) healthy stems, (b) negative control, (c) liquid inducer, (d) gel inducer, (e) paste inducer. tr = trachea, jx = xylem pith radius, tc = tracheid cells, black arrows indicate resin compounds.

3.4. Determination of the Best Producer of Agarwood

The dry weight value was taken after being separated from healthy wood, while the dry weight value was determined after three days of drying at room temperature. Based on the agarwood produced, the wet and dry weight of *kamedangan* and dust were significantly different from the control and among the three inducers (**Fig. 5**). The highest average weight of dry pods was obtained from seedlings after being induced for 75 days, found in *G. versteegii* seedlings, which were inoculated using a gel inducer, namely 1.32 g, where the highest value quantity of dry *kamedangan* was produced. The gel inducer can stimulate stem enlargement around the inoculation hole, caused by the accumulation of fungi in the area (Mohamed et al. 2014; Try et al. 2017).

The control tree with induction treatment using aquadest got the lowest average weight of dry kale, which was 0.03 g (**Fig. 5**). The agarwood formed on the control tree was assumed to be due to physical injury when the stem was perforated using a drill bit. The cause of agarwood is only formed around the inoculation hole and does not form in wounds by stripping the skin (Try

et al. 2017). It indicated that pure water could not induce agarwood formation (Chong et al. 2015). Based on the observation of agarwood thickness by observing the anatomy of the infected stem. It was seen that the control tree had the least number of interxylary phloem cells filled with agarwood resin. It showed that agarwood inducers play an essential role in forming agarwood resin.

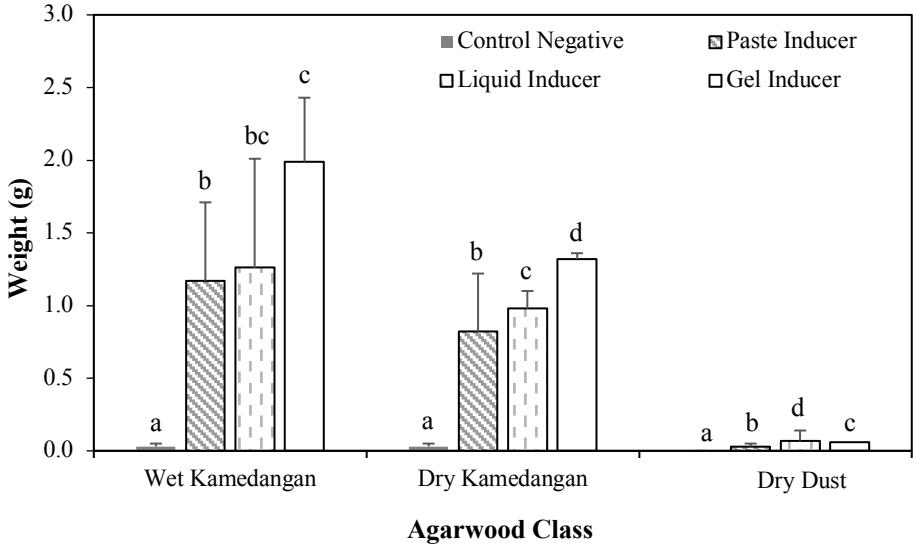

Fig. 5. Histogram of the average weight of agarwood after inoculated with four different inducers aged 75 DAI (Notes: a, b, c, d show a significant difference; bc shows not significantly different).

The transmission of agarwood is characterized by a brown color that spreads toward the top of the inoculation hole and the inoculation hole. The brown zone on the initially white wood indicates the presence of agarwood (Rasool and Mohamed 2016). The length of the agarwood transmission upwards is longer than downwards. It might be caused by the inducer flowing upward through the tracheal cells with water and nutrients flowing from the roots to the leaves. The flowability is influenced by capillary power and transpiration rate in the leaves. In contrast, the inducer downwards is only influenced by the capillary forces and the impulse from the photosynthetic products from the leaves. The longest total length of agarwood transmission (up and down) was found in the liquid inducer, an average of 10.2 cm, followed by the gel inducer at 9.8 cm and the paste at 8.1 cm (**Fig. 6**). This vertical transmission is caused by agarwood resin spread through the xylem vessels (Chong et al. 2015). The liquid form that is more easily absorbed makes the liquid inducer have the longest transmission. It is because liquid substances are more easily absorbed to enter the water transport pathway in the stem. Xylem provides a pathway by minimizing the pressure gradient to make water moving toward the leaves easier (Kim et al. 2014)., After three months of inoculation, the transmission of agarwood in young *Aquilaria malaccensis* plants inoculated with fungi belonging to the class Deuteromycetes and Ascomycetes was between 0.8-1.5 cm (Mohamed et al. 2014).

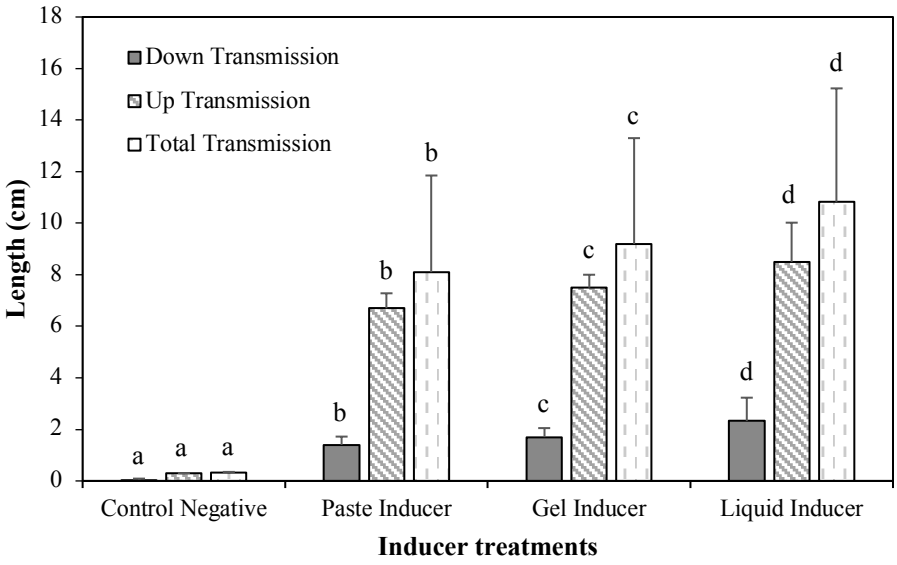

Fig. 6. Histogram of transmission of agarwood inoculated by *G. versteegii* at the seedling stage, 75 DAI (Notes: a, b, c, d show a significant difference).

4. Conclusions

The results showed that the seedlings of *G. versteegii* could produce agarwood of *kamedangan* class. The longest total agarwood transmission in the vertical direction in the inoculated seedlings was found in the liquid inducer with an average length of 10.2 cm. The type of inducer produced *kamedangan* with the highest average weight was found in the 1.32 g gel inducer. Meanwhile, the liquid inducer produced a better curing quality than other inducers, while the gel and paste inducer produced the highest quantity of *kamedangan*.

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