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Improving Agarwood (*Aquilaria malaccensis* Lamk.) Plantlet Formation Using Various Types and Concentrations of Auxins

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Abstract

Aquilaria malaccensis Lamk. is one of the most widespread agarwood-producing plants that face extinction due to overexploitation. Agarwood propagation using *in vitro* culture techniques is capable of producing large quantities of plants in a shorter time and free from pests and diseases. Therefore, this study was conducted to analyze the effect of auxins type and concentration on agarwood plantlet formation using a split-plot design. The main plot was the type of auxin which included IAA, IBA and NAA, while the subplot was the concentration used which consisted of 0; 5; 10; 15 and 20 µM. The variable observed was agarwood plantlet formation with parameters measured including the number of shoots and leaves, plant height, and number of roots. The results showed that the formation of agarwood plantlets was controlled by the type, concentration, and interaction between the type and concentration of auxin. Furthermore, explants cultured on Murashige Skoog (MS) medium supplemented with 10 µM IBA produced the highest number of shoots (3.39 shoots explant^{-1}) and leaves (7.25 leaves explants^{-1}), while the addition of 10 uM NAA resulted in the highest number of roots (2.52 roots explant^{-1}). This is the first time a study is conducted to evaluate the effect of type and concentration of auxins on agarwood plantlet formation. The production of high-quality shoots and plantlets increased agarwood germplasm availability to prevent extinction and support sustainable production.

Keywords: IAA; IBA; *in vitro* production; NAA; rooting

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INTRODUCTION

Agarwood (*Aquilaria malaccensis* Lamk.) is one of the most exploited woody plants due to its importance in health, religion and aesthetics. In Indonesia, there are many species of agarwood which include *Aquilaria* spp., *Aetoxylontallum* spp., *Gyrinops* spp. and *Gonystylus* spp., that spread from Sumatera, Borneo, and Papua (Santoso et al., 2012). However, the population is shrinking due to deforestation and overexploitation (Rahmat and Nurlia, 2015). Currently, the plant is included in Appendix II of Convention on International Trade in Endangered Species (CITES) (CITES, 2004), where the harvest should be controlled and the export is limited to a quota to maintain its sustainability (Mandang and Wiyono, 2002). These conditions necessitate a proper conservation method and subsequent seedling production.

Agarwood propagation using *in vitro* culture techniques holds promise as this technique is capable of producing large quantities of plants

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in a shorter time and free from pests and diseases (Sulistiani and Yani, 2012). Many studies related to the *in vitro* culture of agarwood have been carried out, including callus induction (Leksonowati et al., 2017; Wahyuni et al., 2020), organogenesis (Borpuzari and Kachari, 2018; Arhvitasari et al., 2019), and shoot induction and propagation (Mulyono, 2010; Wardatutthoyyibah et al., 2015; Azwin, 2016). However, there is no published report on successful plantlet formation of Agarwood using different auxins. This suggests that root induction to produce good plantlets is an important problem to be resolved.

Fauzan et al. (2015) reported that combination between IBA and kinetin cannot induce rooting of agarwood (*Aquilaria beccariana* van Tiegh.). Wardatutthoyyibah et al. (2015) also reported that the combination of BAP and NAA showed no significant results on *A. malaccensis* Lamk. rooting. Furthermore, the induction of agarwood root had previously been carried out by Listiana et al. (2018) and Munasinghe et al. (2021). Listiana et al. (2018) reported that NAA was better than IBA in inducing *Aquilaria filaria* rooting. Meanwhile, Munasinghe et al. (2021) reported that NAA independently induced the roots of agarwood *Gyrinops walla*. Therefore, the induction of agarwood roots using various types and concentrations of auxin is essential.

Root induction is one of the essential steps in plantlet formation in *in vitro* culture. Optimization of *in vitro* adventitious root formation contributes to the development of a large-scale production system (Li et al., 2021). Rooting is controlled by both internal and external factors (Kumsa, 2020) such as different physiological, biochemical, and genetic factors, medium composition, plant growth regulators and physical growth factors (Arab et al., 2018). This process is also strongly influenced by auxins, vitamins, amino acids, sucrose, pH, activated carbon (AC) and induction time in the dark (Li et al., 2021).

Auxin is a class of plant growth regulator that promote induction and development of root, including lateral root formation. It accelerates root growth, increases the number of roots, and improves the root system of explants (Aloni, 2010; Muller and Leyser, 2011; Pop et al., 2011). Auxins used here were Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA). IAA has slightly unstable properties compared to other auxins since it is easily degraded by light or oxidative enzymes (Park et al., 2017; Kumar et al., 2019). IBA and NAA are more effective than IAA because they are more stable and less sensitive to oxidation and light (Zaerr and Mapes, 1982; Feng et al., 2012). According to Salisbury and Ross (1995), NAA is not easily degraded and active for a long time, but it has a narrow concentration range. Meanwhile, IBA has a more flexible concentration range (Hendaryono and Wijayani, 2012). Peña-Baracaldo et al. (2018) found that IBA application improved rooting percentage, root volume and decreased the percentage of losses of *Leucadendron* sp. According to Li et al. (2021), a higher rooting rate, survival rate, and average root number were obtained on the medium containing 2.0 mg l^{-1} NAA and 0.25 mg l^{-1} IBA in hybrid larch. Culturing mulberry shoots on MS medium supplemented with 1 mg l^{-1} NAA enhanced growth and development of the root system, and successfully acclimatized (Taha et al., 2020). A good rooting system is needed to increase the success of the acclimatization processes and subsequent plant establishment in the soil.

This research was conducted to analyze the effect of auxin type and concentration on agarwood plantlet formation. Plantlets with superior rooting systems increase the success of the acclimatization process. The production of high-quality plantlets enables agarwood conservation as well as improves its sustainable production.

MATERIALS AND METHOD

Plant material

The plant materials used were agarwood shoots which previously cultured on multiplication medium consisting of Murashige-Skoog (MS) medium (Sigma-Aldrich M5519) supplemented with 20 g l^{-1} sucrose and 5 µM BAP (Sigma-Aldrich B3408) and solidified with 0.25% phytagel (Sigma-Aldrich P8169).

Root induction and plantlet formation

Agarwood root induction and plantlets formation were conducted on MS medium supplemented with 20 g l^{-1} sucrose and solidified with 2.5 g l^{-1} phytagel. The effect of IAA (Sigma-Aldrich I2886), IBA (Sigma-Aldrich I5386) and NAA (Sigma-Aldrich N0640) at various concentrations were studied. The study was conducted experimentally using a split-plot design with three replications. The main plots

were the types of auxin consisting of IAA, IBA and NAA, while the subplots were auxin concentrations at 0 µM, 5 µM, 10 µM, 15 µM and 20 µM. The cytokinin concentrations used are modified from the results reported previously by Wardlisatutthoyyibah et al., (2015); Azizi et al. (2017); Prasetyo et al., (2020); and Wahyuni et al., (2020) and the medium pH was adjusted and set to 5.8. The micro shoots were cultured (1 explant bottle^{-1}) and incubated at room temperature of 24°C under continuous light for 12 weeks. The variable observed was agarwood plantlet formation and the parameters measured include the number of shoots, number of leaves, plant height and number of roots.

Data analysis

The data obtained were analyzed using an analysis of variance (ANOVA), followed by the Honestly Significant Difference (HSD) tests with a confidence level of 95%.

RESULTS AND DISCUSSION

An ex-situ agarwood conservation effort was conducted by producing plantlets *in vitro* using MS media supplemented with several auxins, such as IAA, IBA and NAA. This is the first study is conducted to evaluate the effect of type and concentration of auxin on agarwood plantlet formation. During this study all agarwood explants grew well in all treatment media, as seen in the increased explant size, the number of shoots (1 to 15 shoots), leaves (0 to 65 leaves) and roots (0 to 9 roots) formed, at 12 weeks. The increased size is an indication of cell division and enlargement, while the emergence of new shoots indicated cell differentiation processes (Prasetyo et al., 2020). Furthermore, the results also showed that addition of growth regulators increases plant growth (Gultom et al., 2012). Both exogenous and endogenous growth regulator application determines the direction of culture development (Ivanchenko et al., 2010; Park et al., 2017), which can have different effects according to the concentration (Saini et al., 2013).

An interesting discovery was that shoots still grew well even in the media without cytokinin application. It is suggested that high content of endogenous cytokinins in the shoot might have controlled explants' growth. In this research, the explants used were micro shoots derived from a culture in MS media supplemented with 5 µM BAP. Long exposure to BAP increased endogenous BAP level, since it is easily conjugated with glucose to become the storage form (Friml, 2003; Sauer et al., 2013). BAP is easily absorbed and translocated in the form of 9, β-D-Ribofuranosyl-BAP (9R-BAP) and stored as 3,β-D-Glucopyranosyl-BAP (3G-BAP) and 9, β-D-Glucopyranosyl-BAP (9G-BAP). This stored BAP can be hydrolyzed by β-Glucosidase enzyme to produce free and active BAP (Reinert and Yeoman, 1982; Schaller et al., 2014; Feng et al., 2017). The interaction between endogenous cytokinins and exogenous auxins stimulate growth and differentiation of agarwood shoots. Agulló-Antón et al. (2011); Feng et al. (2012); Schaller et al. (2014) reported that the interaction between cytokinins and auxins induces lateral shoots. Shoot formation can be stimulated by manipulating the concentrations of auxin and cytokinins (Lestari, 2011; Sanan-Mishra et al., 2013; Admojo and Prasetyo, 2018).

The HSD test results on the effect of different auxin types on agarwood plantlet formation (Table 1) showed that IAA treatment produced the highest number of shoots and leaves formed, i.e. 1.96 shoots explant^{-1} and 5.14 leaves explant^{-1}, respectively. However, IAA was not significantly different from IBA (Figure 1). The average number of shoots produced (1.96 shoots explant^{-1}) is slightly higher than the report of Fauzan et al. (2015), but lower than that reported by Karlianda et al. (2013). About 1.91 shoots explant^{-1} were obtained from shoot treated with the combination of IBA 0 mg l^{-1} and BAP 0.03 mg l^{-1} (Fauzan et al., 2015), while Karlianda et al. (2013) obtained 11 buds explant^{-1} after treatment with 0.1 mg l^{-1} NAA and 2.5 mg l^{-1} BAP. Furthermore, there was also a correlation between the number of the shoot and the number of leaves. The number of shoots is directly proportional to the leaves formed. These results are consistent with results reported by Prasetyo et al. (2020), where the increased number of shoots is accompanied by the number of leaves.

Table 1 also showed that the highest average of plant height was shown by explant cultured on the media supplemented with IBA, with plant height reaching 1.98 cm, but there is no significant difference with that of IAA. This average plant height was higher than the report of Mulyono (2010) who obtained an average height of 1.7 cm under 0.1 mg l^{-1} IBA + 0.05 mg l^{-1} BAP. Fauzan

et al. (2015) reported that the addition of IBA at a concentration higher than BAP concentration produced higher average of stem internodes. Meanwhile, it was also found that the addition of NAA resulted in the lowest average height but produced the highest number of roots (1.18 roots explant^{-1}) (Figure 1c). These results indicated that NAA is more effective in inducing root formation, as also ever reported by Mller and Leyser (2011) and Simon and Petrášek (2011).

Table 1. The effect of different type of auxin on plantlet formation of agarwood. The values shown are means with n =15

Types of auxins	Number of shoots	Number of leaves	Plant height	Number of roots
IAA	1.97 ± 0.16^{a}	5.14 ± 0.57^{a}	1.95 ± 0.07^{a}	0.81 ± 0.10^{b}
IBA	1.92 ± 0.42^{a}	4.82 ± 0.83^{a}	1.98 ± 0.13^{a}	0.95 ± 0.17^{b}
NAA	1.30 ± 0.25^{b}	2.62 ± 1.09^{b}	1.43 ± 0.14^{b}	1.18 ± 0.35^{a}

Note: Values followed by different letters show significant difference in HSD (≤ 0.05)

Figure 1. The appearance of plantlets under different types of auxins: (a) plantlet on MS medium with IAA 10 µM; (b) plantlet on MS medium with IBA 10 µM; (c) plantlet on MS medium with NAA 10 µM

The HSD test results on the effect of different concentrations of auxin on plantlet formation (Table 2) showed that 10 µM auxin resulted in a significantly higher number of shoots and roots, which were 2.13 shoots explant^{-1} and 1.31 roots explant^{-1}, respectively. The number of shoots produced was not significantly different from that of treated with 0 µM and 5 µM auxin. The average number of shoots produced here is slightly higher than the previous results published by Fauzan et al. (2015) i.e. 1.91 shoots explants^{-1}. However, it is lower than the report of Karlianda et al. (2013), which was 11 shoots explant^{-1}. The addition of auxin above 10 µM resulted in lower number of shoots. Aziz et al. (2017) and Karlianda et al. (2013) reported that higher concentration of auxin inhibits both shoot and root formation.

Table 2. The effect of different concentration of auxin on plantlet formation of agarwood. The values shown are means with n = 9

Concentrations of auxin (µM)	Number of shoots	Number of leaves	Plant height	Number of roots
0	2.01 ± 0.33^{a}	5.89 ± 1.05^{a}	1.96 ± 0.14^{a}	0.82 ± 0.13^{b}
5	1.90 ± 0.38^{a}	4.51 ± 1.13^{b}	1.77 ± 0.23^{ab}	0.84 ± 0.22^{b}
10	2.13 ± 0.62^{a}	4.83 ± 1.46^{b}	1.66 ± 0.15^{b}	1.31 ± 0.54^{a}
15	1.28 ± 0.22^{b}	2.75 ± 0.93^{c}	1.74 ± 0.27^{b}	1.05 ± 0.26^{ab}
20	1.34 ± 0.20^{b}	2.99 ± 0.89^{c}	1.81 ± 0.22^{ab}	0.88 ± 0.15^{b}

Note: Numbers followed by different letters show significant difference in HSD (≤ 0.05)

According to Table 2, explants cultured on media without auxin (0 µM) resulted in the highest number of leaves and plant height, which was 5.89 leaves explant^{-1} and 1.96 cm explant^{-1}. The average plant height obtained was higher than the report of Mulyono (2010),

where an average height of 1.7 cm was obtained. These results indicated that endogenous auxin in plants stimulates cell elongation leading to longer shoot and more leaves formed. Furthermore, exogenous auxins at a concentration between 10 µM to 15 µM improved agarwood rooting. Up to a certain point, high auxin concentrations increase root formation (Aloni, 2010; Kazan, 2013). In general the use of low concentration of growth regulator stimulates growth and development of explants, but at high concentrations inhibits explant morphogenesis (Shakirova et al., 2010; Pacurar et al., 2014; Fattorini et al., 2017; Prasetyo et al., 2020).

HSD test results on the effect of interaction between type and concentration of auxin (Table 3) showed that the highest number of shoots and leaves were produced by explants grown at media supplemented with 10 µM IBA with an average of 3.39 shoots explant^{-1} and 7.25 leaves explants^{-1} (Figure 2). The average number of shoots produced is slightly higher than the report of Fauzan et al. (2015), but lower than that of Karlianda et al. (2013). Shoot and leaf formation occurred sequentially and the correlation analyses confirmed the significant relationship between shoot and leaf formation ($r = 0.924$; P-value < 0.01; $n = 45$). Arhvitasari et al. (2019) also showed that the number of leaves is influenced by the number of shoots formed. Furthermore, the addition of 20 µM IBA resulted in the highest average shoot height (2.16 cm). These results indicated that IBA is more effective in stimulating shoot induction and subsequent growth, which is consistent with the results of Peña-Baracaldo et al. (2018), Oakes et al. (2020), and Li et al. (2021).

Figure 2. The appearance of best selected agarwood shoots after 12 weeks of culture in MS medium: (a) under 10 µM IBA; (b) without NAA (0 µM); (c) without IAA (0 µM); (d) under 5 µM IBA

Table 3. The effect of types and different concentration of auxin on plantlet formation of agarwood. The values shown are means with n = 3

Treatment (µM)	Number of shoots	Number of leaves	Plant height	Number of roots
IAA 0	2.37 ± 0.14^{b}	6.90 ± 0.52^{a}	1.96 ± 0.04^{ab}	0.71 ± 0.00^{c}
IAA 5	2.14 ± 0.21^{bc}	5.07 ± 0.23^{bcd}	1.95 ± 0.11^{ab}	0.71 ± 0.00^{c}
IAA 10	1.99 ± 0.15^{bcd}	5.54 ± 0.29^{bc}	1.95 ± 0.08^{ab}	0.71 ± 0.00^{c}
IAA 15	1.72 ± 0.17^{cde}	4.47 ± 0.32^{cde}	1.99 ± 0.15^{ab}	0.88 ± 0.17^{bc}
IAA 20	1.63 ± 0.11^{def}	3.70 ± 0.63^{ef}	1.91 ± 0.09^{ab}	1.05 ± 0.17^{bc}
IBA 0	1.28 ± 0.14^{efg}	3.60 ± 0.34^{ef}	1.96 ± 0.27^{ab}	1.05 ± 0.17^{bc}
IBA 5	2.43 ± 0.24^{b}	6.27 ± 0.63^{ab}	2.08 ± 0.11^{a}	0.71 ± 0.00^{c}
IBA 10	3.39 ± 0.30^{a}	7.25 ± 0.55^{a}	1.63 ± 0.05^{bc}	0.71 ± 0.00^{c}
IBA 15	1.14 ± 0.14^{fg}	2.89 ± 0.25^{fg}	2.08 ± 0.04^{a}	1.39 ± 0.35^{b}
IBA 20	1.38 ± 0.21^{efg}	4.09 ± 0.46^{def}	2.16 ± 0.08^{a}	0.88 ± 0.17^{bc}
NAA 0	2.38 ± 0.07^{b}	7.17 ± 0.18^{a}	1.96 ± 0.04^{ab}	0.71 ± 0.00^{c}
NAA 5	1.14 ± 0.14^{fg}	2.17 ± 0.48^{gh}	1.29 ± 0.06^{d}	1.10 ± 0.39^{bc}
NAA 10	1.00 ± 0.00^{g}	1.71 ± 0.33^{ghi}	1.41 ± 0.10^{cd}	2.52 ± 0.29^{a}
NAA 15	1.00 ± 0.00^{g}	0.88 ± 0.17^{i}	1.15 ± 0.07^{d}	0.88 ± 0.17^{bc}
NAA 20	1.00 ± 0.00^{g}	1.17 ± 0.25^{hi}	1.35 ± 0.06^{cd}	0.71 ± 0.00^{c}

Note: Numbers followed by different letters show significant difference in HSD (≤ 0.05)

Table 3 also showed that the highest plant height was measured on the explant cultured on 15 µM IBA (2.08 cm shoot^{-1}). However, it was not significantly different from the results obtained by explant cultured on 20 µM IBA. The average plant height obtained was higher than the report of Mulyono (2010) (1.7 cm shoot^{-1}). Fauzan et al. (2015) observed that agarwood plant (*A. beccariana* van Tiegh.) height increased with increasing IBA concentration. IBA plays a role in cell elongation (Fattorini et al., 2017; Sofian et al., 2018). Auxins stimulate cell elongation by affecting cell wall metabolism and properties to deposit several primary cell wall materials at both ends of the cell. Stretched cell wall structure allows more cell wall deposits (Jin Feng et al., 2012; Zhao, 2014; Kaur and Kapoor, 2016).

The addition of 10 µM of NAA resulted in the highest average number of roots (2.52 roots explant^{-1}) and was significantly different from all other treatments (Figure 3a). This finding was higher than the report of Listiana (2018) and Munasinghe (2021) who used the same NAA concentration (2 mg l^{-1}). Listiana (2018) obtained 2.5 roots explant^{-1} of *A. filaria*, whereas Munasinghe (2021) got 1.6 roots explant^{-1} of *G. walla* (Srilangka agarwood). NAA was considered to be the best auxin for root induction, as reported by Listiana (2017) and Taha et al. (2020). Listiana (2017) reported that the application of 2 mg l^{-1} NAA (equivalent to 10.74 µM) produced an average of 2.5 roots explant^{-1}. NAA is a synthetic auxin which is more stable than others (Zaerr and Mapes, 1982; Gunawan, 1992). Furthermore, It has a slow translocation and low activity so that it will be more concentrated in locations where it is applied. It will be concentrated at the base of the explants, and stimulate the explants to form roots (Zaerr and Mapes, 1982; Kumlay, 2014).

Some of the explants formed callus at shoot base instead of root (Figure 3b). Callus is formed when auxins and cytokinins are available in the explants at a balance concentration. This fact strengthens the indication that explants contain sufficient endogenous cytokinins which interact with auxin treatment to stimulate callus induction and growth at the base of shoot. The interaction between cytokinins and auxins at the same concentration in the wounding area induce callus formation (Ikeuchi et al., 2013; Fehér, 2019).

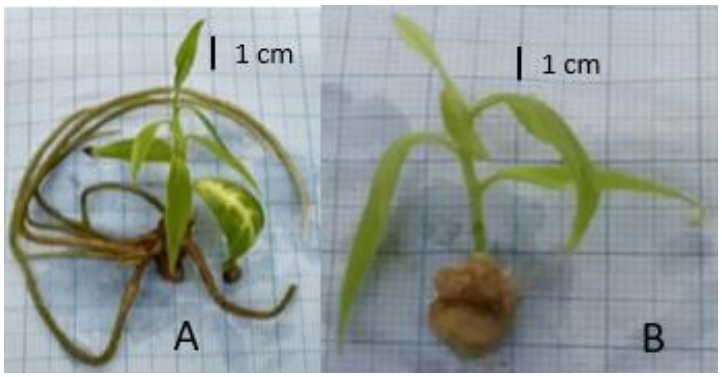

Figure 3. Plantlet and callusing shoot: (a) plantlet on under MS medium supplemented with NAA 10 µM; (b) Callusing shoot under MS medium supplemented with NAA 20 µM

CONCLUSIONS

The formation of agarwood plantlets was controlled by the type, concentration, and interaction between the type and concentration of auxin given. Explants cultured on MS medium supplemented with 10 µM IBA produced the highest number of shoots (3.39 shoots explant^{-1}) and leaves (7.25 leaves explants^{-1}). Meanwhile, the addition of 10 µM NAA resulted in the highest number of root (2.52 roots explant^{-1}). The production of high-quality shoots and plantlets improves agarwood germplasm conservation to prevent the extinction and support sustainable production of agarwood.

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