# COVERING LETTER

**Maulana Tamyiz[1], Sugiyono*[1], Lucky Prayoga[1], Rendie Prasetyo[1]**

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**I. First author:**

1. Name : Maulana Tamyiz
2. Affiliation : Department of Botany, Faculty of Biology, Universitas Jenderal Soedirman
3. E-mail : mmaul87@gmail.com
4. Orcid ID : -
5. Phone number : +62 857-4357-8366

**II. Second author*:**

1. Name : Sugiyono
2. Affiliation : Department of Botany, Faculty of Biology, Universitas Jenderal Soedirman
3. E-mail : gieks_sugiyono@hotmail.com
4. Orcid ID : https://orcid.org/0000-0003-1661-312X
5. Phone number : +62 813-2739-6412

**III. Third author:**

1. Name : Lucky Prayoga
2. Affiliation : Department of Botany, Faculty of Biology, Universitas Jenderal Soedirman
3. E-mail : lucky.prayoga@unsoed.ac.id
4. Orcid ID : https://orcid.org/0000-0001-6581-6068
5. Phone number : +62 812-2721-546

**IV. Fourth author:**

1. Name : Rendie Prasetyo
2. Affiliation : Department of Botany, Faculty of Biology, Universitas Jenderal Soedirman
3. E-mail : rendie.prasetyo@gmail.com
4. Orcid ID : https://orcid.org/0000-0002-4683-9800
5. Phone number : +62 852-2134-6914

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


## Abstract

Agarwood (*Aquilaria malaccensis* Lamk.) is one of the most widely used agarwood-producing plants but face extinction due to overexploitation. This study has been carried out with a view to study the effect of auxins on *in vitro* agarwood rooting, and to determine the best type and concentration of auxin to stimulate agarwood rooting. This research has been conducted experimentally using a split-plot design. The main plot was the type of auxin which included IAA, IBA, and NAA. The sub-plot was the concentration of auxin used which consisted of 5 levels, i.e., 0; 5; 10; 15; and 20 µM. The variable observed was agarwood plantlet formation with parameters measured included the number of shoots, number of 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 given. Explants cultured on MS medium supplemented with 10 µM IBA produced the highest number of shoots (3.39 shoots/explant) and leaves (7.25 leaves/explants). The addition of 10 uM NAA resulted in the highest number of root (2.52 roots/explant). The production of high-quality shoots and plantlets is expected to increase agarwood germplasm availability to prevent agarwood extinction and increase the availability of high-quality seedlings to support sustainable production of agarwood.


**Keywords:** *Rooting, IAA, IBA, NAA.*

## INTRODUCTION

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

Agarwood propagation using *in vitro* culture techniques looks promising, since this technique is capable of producing large quantities of plants in a faster time and free of pests and diseases (Sulistiani & Yani, 2012). Plant *in vitro* culture is defined as the isolation of any plant parts and subsequently cultured on a nutrition medium under sterile conditions with a view to produce new viable plants (George et al., 2008; Pierik, 1982). Several factors control the success of *in vitro* plant propagation, including the media and plant growth regulators used, as well as culture environments (Bhatia & Bera, 2015).

Root induction is one of the essential steps in *in vitro* culture, including in agarwood propagation. Optimization of *in vitro* adventitious root induction 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). Different physiological, biochemical, and genetic factors such as genotype/cultivars, medium composition, plant growth regulators, and physical factors affect rooting (Arab et al., 2018).

Organogenesis in rooting 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 (PGR) which known to promote induction and development of root, including lateral root development. Auxins can accelerate root growth, increase the number of roots, and improve the root system of explants (Aloni, 2010; Mller & Leyser, 2011; Pop et al., 2011). Auxins used in this study 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. It can be degraded by light or broken down by oxidative enzymes (Kumar et al., 2019; Park et al., 2017). IBA and NAA are more effective auxin than IAA because they are more stable to oxidation and light (Jin Feng et al., 2012; Zaerr & Mapes, 1982). According to Salisbury & 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 & Wijayani, 2012).

Arab et al. (2018) reported that type of culture media, Fe-EDDHA and Thiamine are effective factors for *in vitro* rooting of G×N15 prunus rootstock. Meanwhile a study by Peña-Baracaldo et al. (2018) found that IBA improved the rooting percentage, the root volume, and decreased the percentage of losses. Li et al. (2021) found that in hybrid larch, a higher rooting rate, survival rate, and average root number were obtained with medium containing 2.0 mg/L NAA and 0.25 mg/L IBA. In this study, adding 5 mg. $L^{-1}$ Glutamic acid to the culture medium had the best effect on rooting, and the survival rate of shoots reached 90%. In addition, 7.5 g. $L^{-1}$ sucrose produced the highest average root number. The rooting effect was best when the pH of the medium was 6.7.

A good rooting system is needed to increase the success of the acclimatization processes. Several factors have been reported affecting of the success of acclimatization stage including: the condition of the plantlets, light intensity, temperature, relative humidity, types of planting media, nutrition and fertilizing and controlling of pest and microorganisms (Irsyadi, 2021). Culturing mulberry shoot on the MS medium supplemented with 1 mg/l NAA enhanced growth figures of root system which successfully acclimatized (Taha et al., 2020). The most effective methods for rooting and acclimatization included seven weeks in elongation medium, using Jiffy peat pellets soaked in water as the rooting substrate, cutting off callus while submerged, then dipping in 0.31% IBA rooting gel, and placing plantlets in low light after rooting (Oakes et al., 2020).

This research has been carried out with a view to study the effect of auxins on *in vitro* rooting of agarwood, and to determine the best type and concentration of auxin to stimulate agarwood rooting. Plantlets with great rooting systems can increase the success of the acclimatization process. The production of high-quality plantlets will help agarwood conservation as well as improving sustainable production of agarwood.

## MATERIALS AND METHODS

### Plant material

The plant materials used were agarwood shoots cultured on multiplication medium consisted of Murashige-Skoog (MS) medium (Sigma-Aldrich-M5519) supplemented with 20 $gL^{-1}$ sucrose 5 µM BAP (Sigma-Aldrich - B3408) and solidified with 0.2% phytagel (Sigma-Aldrich P8169). The cytokinin concentration used are modified result of a report by Azizi et al. (2017) and Wahyuni et al. (2020).

### Root induction and plantlet formation

Agarwood root induction and plantlets formation has been carried out on Murashige-Skoog (MS) medium supplemented with 20 $gL^{-1}$ sucrose and solidified with 2.5 $gL^{-1}$ phytagel. The effect of with IAA (Sigma-Aldrich - I2886), IBA (Sigma-Aldrich - I5386), and NAA (Sigma-Aldrich - N0640) at various concentrations were studied. The study has been carried out experimentally using a split-plot design with three replications. The main plots were the types of auxin consisting of IAA, IBA, and NAA, whereas the subplots were auxin concentrations at 0 µM, 5 µM, 10 µM, 15 µM and 20 µM. The cytokinin concentration used are modified result reported (Prasetyo et al., 2020; Wahyuni et al., 2020; Wardatutthoyyibah et al., 2015). The medium pH was adjusted and set tot 5.83. The explants were culture (1 explant/bottle) and incubated at room temperature of 24°C under continuous light for 12 weeks. The variable observed was agarwood plantlet formation with parameters measured including 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 efforts have been carried out by producing plantlets *in vitro* using MS media supplemented with several auxins, namely IAA, IBA, and NAA. During this study all agarwood explants grew well in all treatment media, as seen in the number of shoots, leaves, and roots formed in each treatment, at 12 weeks after planting. The size of the explants on the treatment media swelled/increased. Explant swelling indicated cell division and enlargement, while the emergence of new shoots indicated cell differentiation processes (Prasetyo et al., 2020). This finding also indicated the addition of growth regulators can increase plant growth (Gultom et al., 2012). Exogenous growth regulators application and those produced by plants endogenously determine the direction of culture development (Ivanchenko et al., 2010; Park et al., 2017). The response to growth regulators can differ in plant types due to the difference in endogenous hormones concentration (Saini et al., 2013).

There was also interesting discovery in this study that shoot still grew nicely even in the media without cytokinin applications. This result indicated that high content of endogenous cytokinins in the

shoot might have controlled the explants growth. The explant used in this study were microshoots derived from a culture in MS media supplemented with 5 µM BAP. Long exposure to BAP could increase endogenous BAP level. BAP is a cytokinin which is easily conjugated with glucose which later becomes BAP storage (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). These stored BAP will be easily hydrolyzed by β-Glucosidase enzyme to produce free and active BAP (Jian Feng et al., 2017; Reinert & Yeoman, 1982; Schaller et al., 2014). It is suggested that the interaction between endogenous cytokinins and exogenous auxins stimulate growth and differentiation of agarwood shoots. Agulló-Antón et al. (2011); Jin Feng et al. (2012); Schaller et al. (2014) reported that the interaction between cytokinins and auxins can induce lateral shoots. Shoot formation can be stimulated by manipulating the concentrations of auxin and cytokinins given (Admojo & Prasetyo, 2018; Lestari, 2011; Sanan-Mishra et al., 2013).

The HSD test results on the effect of different auxin types on agarwood plantlet formation (Table 1) showed that IAA treatment resulted in the highest number of shoots and leaves formed, i.e., 1.96 shoots/explant and 5.14 leaves/explant, respectively, although IAA was not significantly different to IBA treatment. There was a correlation between the number of the shoot and the number of leaves. The higher the number of shoots, the greater number of leaves formed (Figure 1). These results are consistent with results reported by Prasetyo et al. (2020) that increased number of shoots is accompanied by an increased number of leaves. Furthermore, Table 1 also showed that the highest average plant height was shown by the addition of IBA with plant height reaching 1.98 cm, and there is no significant difference with that of IAA. Fauzan et al. (2015) reported that the addition of IBA, with a higher concentration than BAP, produced a higher average of stem internodes. Meanwhile, the addition of NAA showed the lowest average height, but produced the number of roots (1.18 roots/explant) (Figure 1C). These results indicated that NAA is more effective in inducing root formation, as also reported by Mller & Leyser (2011) and Simon & Petrášek (2011).

Table 1. The effect of different type of auxin on plantlet formation of agarwood

| Types of Auxins | Number of Shoots | Number of Leaves | Plant Heigh | Number of Roots |
|---|---|---|---|---|
| IAA | 1.96[a] | 5.14[a] | 1.95[a] | 0.81[b] |
| IBA | 1.92[a] | 4.82[a] | 1.98[a] | 0.95[b] |
| NAA | 1.30[b] | 2.62[b] | 1.43[b] | 1.18[a] |

Note: Numbers 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 result on the effect of different concentrations of auxin on plantlet formation (Table 2) showed that auxin at a concentration of 10 µM resulted in a significantly better number of shoots and number of roots (Figure 2), which were 2.13 shoots/explant and 1.31 roots/explant, respectively. The number of shoots produced by 10 µM auxin was not significantly different with those of produced by explant treated with 0 µM and 5 µM auxin. The addition of auxin above 10 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. Furthermore, Table 2 also showed that explant cultured on media without auxin (0 µM) resulted in the highest number of leaves and plant height, which was 5.89 leaves/explant and 1.96 cm/explant. These results indicated that endogenous auxin in plants might had been able to stimulate cell elongation leading to longer shoot, and more leaves. The addition of exogenous auxins at a concentration between 10-15 µM improved agarwood rooting. Up to a certain point, high auxin concentrations will increase root formation (Aloni, 2010; Kazan, 2013). In general the addition of low concentration of growth regulator can stimulate growth and development of explants, whereas high concentrations of PGR can inhibit explant morphogenesis (Fattorini et al., 2017; Pacurar et al., 2014; Prasetyo et al., 2020; Shakirova et al., 2010).

Table 2. The effect of different concentration of auxin on plantlet formation of agarwood

| Concentrations of Auxin | Number of Shoots | Number of Leaves | Plant Heigh | Number of Roots |
|---|---|---|---|---|
| 0 µM | 2.00$^{a}$ | 5.89$^{a}$ | 1.96$^{a}$ | 0.82$^{b}$ |
| 5 µM | 1.90$^{a}$ | 4.51$^{b}$ | 1.77$^{ab}$ | 0.84$^{b}$ |
| 10 µM | 2.13$^{a}$ | 4.83$^{b}$ | 1.66$^{b}$ | 1.31$^{a}$ |
| 15 µM | 1.28$^{b}$ | 2.75$^{c}$ | 1.74$^{b}$ | 1.05$^{ab}$ |
| 20 µM | 1.34$^{b}$ | 2.99$^{c}$ | 1.81$^{ab}$ | 0.88$^{b}$ |

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


Figure 2. The appearance of 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.

HSD test result on the effect of interaction between types and concentration of auxin (Table 3) showed that the highest number of shoots and leaves produced by explant grown at media supplemented with 10 µM IBA with an average of 3.39 shoots/explant and 7.25 leaves/explants (Figure 2). Shoot formation seemed to be followed by leaf formation. The correlation analysis between the number of shoots and the number of leaves (Table 4) confirmed the relationship between the number of shoots and the number of leaves formed (r = 0.924. Arhvitasari et al. (2019) showed that the number of leaves is influenced by the number of shoots formed so that the more shoots formed, the more leaves will be 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. These finding is consistent with results reported by Li et al. (2021); Oakes et al. (2020) and Peña-Baracaldo et al. (2018).

Table 3. The effect of types and different concentration of auxin on plantlet formation of agarwood

| Treatment | Number of Shoots | Number of Leaves | Plant Heigh | Number of Roots |
|---|---|---|---|---|
| IAA 0 µM | $2.37^{b}$ | $6.90^{a}$ | $1.96^{ab}$ | $0.71^{c}$ |
| IAA 5 µM | $2.14^{bc}$ | $5.07^{bcd}$ | $1.95^{ab}$ | $0.71^{c}$ |
| IAA 10 µM | $1.99^{bcd}$ | $5.54^{bc}$ | $1.95^{ab}$ | $0.71^{c}$ |
| IAA 15 µM | $1.72^{cde}$ | $4.47^{cde}$ | $1.99^{ab}$ | $0.88^{bc}$ |
| IAA 20 µM | $1.63^{def}$ | $3.70^{ef}$ | $1.91^{ab}$ | $1.05^{bc}$ |
| IBA 0 µM | $1.28^{efg}$ | $3.60^{ef}$ | $1.96^{ab}$ | $1.05^{bc}$ |
| IBA 5 µM | $2.43^{b}$ | $6.27^{ab}$ | $2.08^{a}$ | $0.71^{c}$ |
| IBA 10 µM | $3.39^{a}$ | $7.25^{a}$ | $1.63^{bc}$ | $0.71^{c}$ |
| IBA 15 µM | $1.14^{fg}$ | $2.89^{fg}$ | $2.08^{a}$ | $1.39^{b}$ |
| IBA 20 µM | $1.38^{efg}$ | $4.09^{def}$ | $2.16^{a}$ | $0.88^{bc}$ |
| NAA 0 µM | $2.38^{b}$ | $7.17^{a}$ | $1.96^{ab}$ | $0.71^{c}$ |
| NAA 5 µM | $1.14^{fg}$ | $2.17^{gh}$ | $1.29^{d}$ | $1.10^{bc}$ |
| NAA 10 µM | $1.00^{g}$ | $1.71^{ghi}$ | $1.41^{cd}$ | $2.52^{a}$ |
| NAA 15 µM | $1.00^{g}$ | $0.88^{i}$ | $1.15^{d}$ | $0.88^{bc}$ |
| NAA 20 µM | $1.00^{g}$ | $1.17^{hi}$ | $1.35^{cd}$ | $0.71^{c}$ |

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

Table 4. The Correlation test between the number of shoots and the number leaves

| | Number of Shoots | Number of Leaves |
|---|---|---|
| Number of Shoots | 1 | |
| Number of Leaves | 0,924** | 1 |

Note: Numbers followed by ** has a correlation at the level r table 0.01 (0.641)

Plant height in this study tended to increase with increasing IBA concentration. Fauzan et al. (2015) also observed similar results that by the increase of IBA concentration, plant height tends to increase. 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 so that a lot of primary cell wall materials will be deposited at both ends of the cell. When the cell structure is stretched, it allows more cell wall deposits (Jin Feng et al., 2012; Kaur & Kapoor, 2016; Zhao, 2014)

Meanwhile, the addition of 10 µM of NAA resulted in the highest average number of roots (2.52 roots/explant) and was significantly different to all other treatments (Figure 3). This results indicated that NAA was better than other auxins for root induction, as also reported by Listiana (2017) and Taha et al. (2020). One of the auxin functions is to stimulate root formation in the shoots (Mulyono, 2012). Listiana (2017) reported that the application of NAA 2 $mg.l^{-1}$ (equivalent to 10.74 µM) can induce an average of 2.5 roots per explant with a percentage of 100%. NAA is a synthetic auxin which is more stable than other auxins (Gunawan, 1992; Zaerr & Mapes, 1982). NAA has a slow translocation and low activity so that NAA will be more concentrated in locations where NAA is applied. The NAA given will be concentrated at the base of the explants, which therefore spur the explants to form roots (Kumlay, 2014; Zaerr & Mapes, 1982).

Some of the explant in this study did not form roots, instead there was a callus formation at shoot base (Figure 3) and might be coupled with ethylene accumulation which in turn inhibit root formation. A callus is formed when auxins and cytokinins are available in the explants at a balance concentrations. This fact strengthens the indication that explants contain sufficient endogenous cytokinins to 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 will induce callus formation (Fehér, 2019; Ikeuchi et al., 2013).

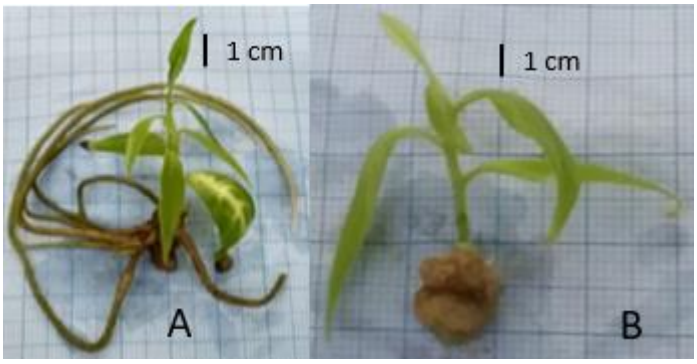


Figure 3. Plantlet and callusing shoot: (A) plantlet on under MS medium supplemented with NAA 10 µM; (B) Callusing shoot under MS medium supplemented with NAA 20 µM

## CONCLUSIONS

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

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