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# The Effect of Media Types and NAA Concentrations on Agarwood (*Aquilaria malaccensis* Lamk.) Shoot Development in *In Vitro* Culture

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## Abstract

Agarwood (*Aquilaria malaccensis* Lamk.) is a forest product commodity with high economic value, but its availability in nature is decreasing due to deforestation and overexploitation. The availability of high-quality seedlings is necessary to meet the demand and conserve the sources of agarwood plants. Therefore, this study investigates the interaction effect between media types and 1-Naphthaleneacetic acid (NAA) concentrations on agarwood shoot development. It also aims to determine the best medium type and NAA concentration to stimulate agarwood shoot development. This research had two stages, namely the shoot induction and development. The shoot induction stage was conducted experimentally, using a completely randomized design (CRD) on a factorial treatment pattern of 2 factors i.e., the types of media and NAA concentrations. The shoots produced from the induction stage were cultured on Murashige-Skoog (MS) media with the same media types but without any NAA addition. Furthermore, the variables observed in both stages were the development of agarwood shoots, and the parameters measured included the number of shoots, number of leaves and shoot lengths. The results showed that the development of agarwood shoots was controlled by the type of medium used. Liquid MS medium supported with filter paper bridge produced the highest average number of shoots with 4.93 and 5.87 shoots explants$^{-1}$ and length at 3.25 and 3.64 cm explant$^{-1}$ in the induction and development stage, respectively. These findings will facilitate mass propagation of agarwood shoots, and in turn the availability of agarwood plantlets supports its conservation and production.

**Keywords:** filter paper bridge; liquid medium; MS media; phytagel; viscous sponge

## *Pengaruh Jenis Media dan Konsentrasi NAA terhadap Perkembangan Tunas Gaharu (Aquilaria malaccensis Lamk.) Secara Kultur In Vitro*

### *Abstrak*

*Gaharu (Aquilaria malaccensis Lamk.) merupakan komoditas hasil hutan yang bernilai ekonomi tinggi, namun ketersediaannya di alam semakin berkurang akibat deforestasi dan eksploitasi berlebihan. Ketersediaan bibit yang berkualitas sangat diperlukan untuk memenuhi permintaan dan melestarikan sumber tanaman gaharu. Oleh karena itu, penelitian ini mengkaji pengaruh interaksi antara jenis media dan konsentrasi 1-Naphthaleneacetic acid (NAA) terhadap perkembangan tunas gaharu. Selain itu juga bertujuan untuk menentukan jenis medium dan konsentrasi NAA yang terbaik untuk merangsang perkembangan tunas gaharu. Penelitian ini terdiri atas dua tahap, yaitu induksi dan pengembangan*

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*tunas. Tahap induksi tunas dilakukan secara eksperimental menggunakan rancangan acak lengkap (RAL) pada pola perlakuan faktorial 2 faktor yaitu jenis media dan konsentrasi NAA. Tunas yang dihasilkan dari tahap induksi dikultur pada media Murashige-Skoog (MS) dengan jenis media yang sama tetapi tanpa penambahan NAA. Selanjutnya variabel yang diamati pada kedua tahap tersebut adalah perkembangan tunas gaharu dan parameter yang diukur meliputi jumlah tunas, jumlah daun dan panjang tunas. Hasil penelitian menunjukkan bahwa perkembangan tunas gaharu dipengaruhi oleh jenis media yang digunakan. Medium MS cair yang dikombinasikan dengan jembatan kertas saring menghasilkan rata-rata jumlah tunas tertinggi sebesar 4,93 dan 5,87 tunas eksplan$^{-1}$ dan panjang masing-masing pada 3,25 dan 3,64 cm eksplan$^{-1}$ pada tahap induksi dan pengembangan. Temuan ini akan memudahkan perbanyakan tunas gaharu secara massal dan pada akhirnya ketersediaan planlet gaharu dapat mendukung konservasi dan produksinya.*

***Kata kunci**: phytagel; jembatan kertas saring; media cair; media MS; spons*

## INTRODUCTION

*Aquilaria malaccensis* agarwood is a forest product with a very high economic value. It is widely used as raw material for perfume, soap, incense, cosmetics and medicines (Gultom et al., 2012; Azwin et al., 2006; Kosmiatin et al., 2016). The prospect of the agarwood market is increasing with the development of society and industry. Indonesia's agarwood export reached more than 2.5 million kilograms between 1996 and 2015 (CITES, 2004). Furthermore, the increase in agarwood demand has resulted in the exploitation of the plants, leading to the decrease of its population in nature (Rahmat and Nurlia, 2015). These plants are propagated conventionally to meet increasing market demand while conserving them in nature, but it takes longer and has a low success rate (He et al., 2005; Samanhudi et al., 2021; Satria et al., 2021). The *in vitro* culture technique can be used to produce large numbers of agarwood plants, free from pathogens and diseases, as well as being economically efficient (Pierik, 1982; Trigiano and Gray, 2004; George et al., 2008).

The explant growth and differentiation in *in vitro* culture are controlled by several factors including the plant's genetic makeup, nutrients, physical growth factors and plant growth regulators (PGR) (Pierik, 1982). Shoot development is an essential step in *plantlet* production. Shoot development is influenced by several factors, including growth regulators and the type of medium used. NAA (1-Naphthaleneacetic acid) is a synthetic auxin commonly used for root induction in plant *in vitro* culture (Zaerr and Mapes, 1982; Kumlay, 2014; Listiana, 2017; Taha et al., 2020). NAA can accelerate seedling growth, root formation and extension from fibrous roots and encourages shoot cell elongation (Mahadi et al., 2015). In addition, NAA does not oxidize quickly and is more stable than natural auxins (Pierik, 1982; George et al., 2008).

The media type commonly used in *in vitro* culture are solid, semi-solid and liquid media (Mbiyu et al., 2012; Alkhateeb and Alturki, 2014; Rezali et al., 2017). These media types affect the osmolarity of the solution it contains and oxygen availability for the growth of cultured explants (Basri, 2016). A liquid medium is considered a better type because it has a high-water potential, hence, there is an easier and faster transportation of water and soluble materials to the plants (George et al., 2008). In addition, explant supporting materials are also essential since it provides enough oxygen for their metabolic processes and growth (Marlin, 2009).

Several studies were conducted related to agarwood plants *in vitro* culture, including works by Nadeak et al. (2012); Julianti et al. (2013); Saikia and Shrivastava (2015); Wardatutthoyyibah et al. (2015); and Borpuzari and Kachari (2018). Fauzan et al. (2015) reported that Indole-3-butyric acid (IBA) and kinetin could not induce *Aquilaria beccariana* rooting. Wardatutthoyyibah et al. (2015) stated that 6-Benzylaminopurine (BAP) and NAA treatment did not significantly affect *A. malaccensis* rooting. Furthermore, Listiana et al. (2018) reported that NAA was the best auxin to promote the rooting of *Aquilaria filaria*. This study was conducted to analyze the effect of the medium types and NAA concentrations on agarwood shoot development. It also determines the best medium

and concentrations for this development. This is the first time the effects of the interaction between medium types and NAA concentrations on *A. malaccensis* shoot development have been studied. Therefore, this study aims to accelerate and increase the production of agarwood seedlings free from diseases and support the conservation efforts of *A. malaccensis.*

## MATERIAL AND METHODS

### Plant material

The plant materials used were micro shoots produced from a study by Tamyiz et al. (2022) and maintained at Laboratory of Plant In Vitro Culture, Faculty of Biology, Universitas Jenderal Soedirman. They were maintained on Murashige-Skoog (MS) medium (M5519 Sigma-Aldrich St. Louis, MO 63118, USA) supplemented with 15 µM BAP (B3408-Sigma-Aldrich St. Louis, MO 63118, USA) and 20 g $l^{-1}$ sucrose and solidified with 0.25% phytagel (P8169-Sigma-Aldrich St. Louis, MO 63118, USA). Previously, agarwood explants were subcultured on an MS medium containing 20 g $l^{-1}$ sucrose, supplemented with 5 µM BAP and solidified with 0.25% phytagel for 12 days to obtain uniform explants.

### Shoot induction

This study involves two stages i.e., shoot induction and development. First, the effect of the medium types and various concentrations of NAA (N0640-Sigma-Aldrich St. Louis, MO 63118, USA) on shoot induction was studied. This stage was conducted on an MS medium supplemented with 20 g $l^{-1}$ sucrose using a completely randomized design on a factorial treatment pattern with two factors. The first factor was the types of medium consisting of MS medium supplemented with 2.5 g $l^{-1}$ phytagel; liquid MS medium with filter paper bridge and liquid MS medium with viscose sponge. The second factor was the concentration of NAA consisting of 5 levels: 0 µM; 2 µM; 4 µM; 6 µM; and 8 µM. All treatment combinations were replicated three times which resulted in 45 experimental units. The NAA concentrations used were a modification of previous studies by Wardatutthoyyibah et al. (2015); Prasetyo et al. (2020); and Tamyiz et al. (2022). The medium pH was adjusted and set to 5.8 before autoclaving to optimize nutrient absorption (Skirvin et al., 1986; Feng et al., 2017). Subsequently, micro shoots were inoculated the treatment medium, one micro shoot per bottle. The cultures were then incubated for 12 weeks at 24°C under continuous light. The variable observed was the formation/emergence of agarwood shoots, and the parameters measured included the number of shoots and leaves, as well as shoot length.

### Shoot development

The shoot development was stimulated in the experiment's second stage, which was conducted for 8 weeks at 24°C under continuous light. The shoots from the induction stage were sub-cultured onto MS basal medium supplemented with 20 g $l^{-1}$ sucrose. The same types of supporting material, namely phytagel, filter paper bridge and viscose sponge were used, without the addition of NAA. The variable observed was the induction of agarwood shoots, and the measured parameters included the number of shoots, leaves and shoot length.

### Data compilation and analysis

The data obtained, including the number of shoots, leaves and shoot length, were analyzed with analysis of variance (ANOVA), followed by the duncan multiple range test (DMRT) at a 95% confidence level, using an SPSS version 16 software.

## RESULT AND DISCUSSION

After 12 weeks of culture, it was discovered that the growth of agarwood explants was diverse, as shown in the increased number of shoots and leaves formed, as well as the length of shoot measured. It was also observed that no proper roots were formed even when NAA was used, which might be related to BAP accumulation in the explant from the previous culture stage. Exposure to BAP for a long-time result in increasing its concentration in the explants. These phenomena were also reported in Tamyiz et al. (2022). BAP is a PGR that is easily absorbed and translocated (Reinert and Yeoman, 1982; Blakesley et al., 1991; Schaller et al., 2014, 2015; Feng et al., 2017), as well as subsequently conjugated with glucose (Friml, 2003; Sauer et al., 2013).

Table 1 shows the analysis of variance results of the effect of medium types, NAA concentration and their interaction on agarwood shoot induction. The data indicated that media type significantly affected the number of shoots formed and their

length. Figure 1 illustrates the appearance of the shoot in different media types but at the same NAA concentration. Table 1 also proved that NAA concentrations and their interaction with media type do not significantly affect the parameters measured. Sufficient endogenous growth regulators caused these conditions in the explants, such as BAP accumulated from the previous culture. BAP is a cytokinin that plays a role in stimulating shoot growth (Erawati et al., 2020), but it is toxic and inhibits plant growth at high concentrations (Gethami and Sayed, 2020). The addition of exogenous growth regulators, which did not significantly affect the growth of explants, has also been reported by (Muliati et al., 2016) in *Sansevieria macrophylla*. Therefore, balancing auxin and cytokinin concentrations is necessary since their interaction at optimal concentrations regulates shoot and root growth (Mahadi et al., 2015).

Table 1. Analysis of the results of the variance of the effect of medium types, NAA concentration and their interaction on the induction of agarwood shoots

| Source of variance | Number of shoots | Shoot length (cm) | Number of leaves |
|---|---|---|---|
| Media type | 6.502* | 6.630* | 0.584 |
| NAA concertation | 0.342 | 1.750 | 0.162 |
| Interaction media type x NAA concentration | 1.311 | 0.630 | 0.354 |

Note: (*) has a significant effect

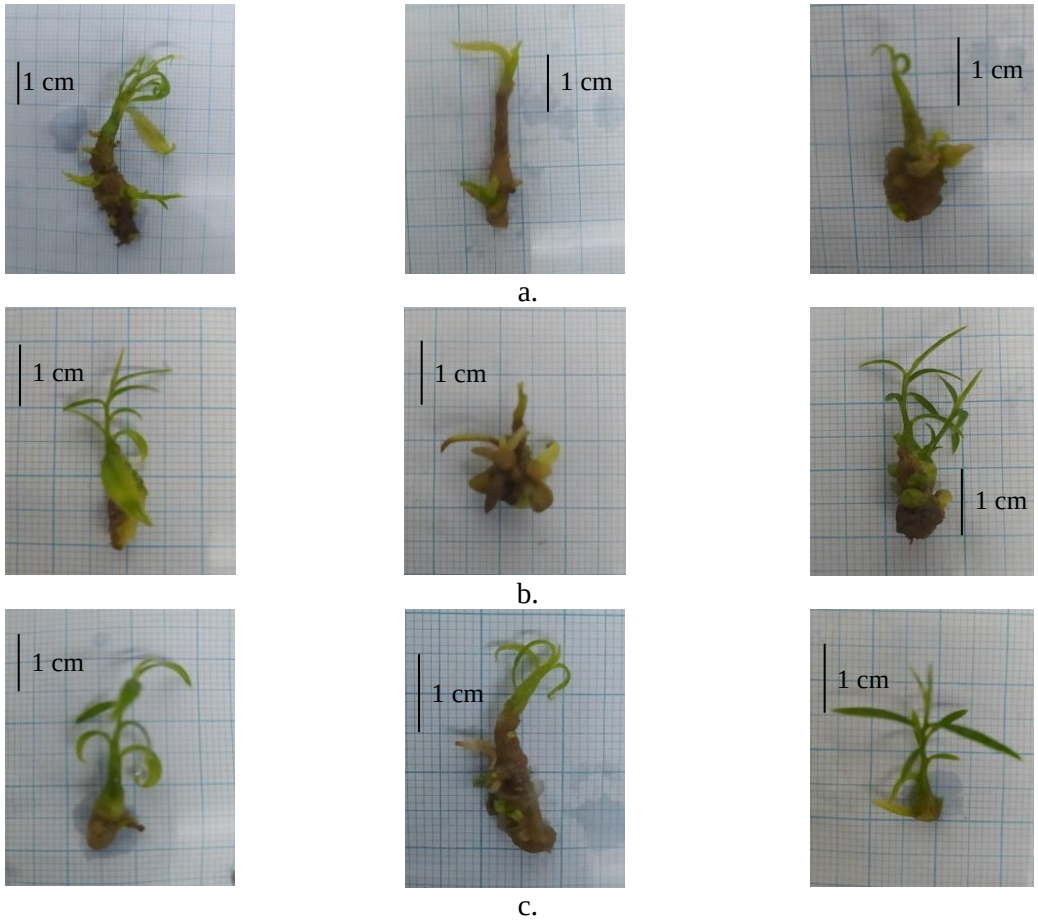


Figure 1. Agarwood shoot appearance at shoot induction state on different media type, (a) shoot grown on liquid MS supported with filter paper bridge and supplemented with 2 µM NAA; (b) agarwood shoot grown on MS medium solidified with 0.25% phytagel and supplemented 2 µM NAA; (c) agarwood shoot grown on liquid MS supported with viscose sponge and supplemented with 2 µM NAA

Furthermore, DMRT test results showed that liquid MS with filter paper bridge produced the highest average number of shoots formed with 4.93 shoots explants$^{-1}$ and length of 3.25 cm explant$^{-1}$. They were significantly different from those produced by explants cultured on liquid MS with viscose sponge and MS medium solidified with 0.25% phytagel (Table 2). These results indicated that liquid MS medium with filter paper bridge supported the growth of explants by providing the necessary but not excessive nutrients and water (waterlogged). The same outcome was reported by Marlin (2009); Grzegorczyk-Karolak et al. (2017) and Nuryadin et al. (2017). Furthermore, the best growth of explants was discovered in a liquid medium supported with a filter paper bridge was caused by an optimal flow of nutrients to the explants (Marlin, 2009). According to Grzegorczyk-Karolak et al. (2017), the nutrients in the liquid medium are evenly distributed, hence, the explants can quickly absorb the nutrients. Additionally, George et al. (2008) stated that a liquid medium accelerates the transport of water and nutrients to plant tissues due to its high potential.

Table 2. DMRT test result on the effect of medium type on the number of shoots and shoot length (n = 15)

| Treatment | Number of shoots | Shoot length (cm) |
|---|---|---|
| Liquid MS supported with viscose sponge | 2.13$^{a}$ | 2.33$^{a}$ |
| MS medium solidified with 0.25% phytagel | 3.13$^{a}$ | 2.71$^{a}$ |
| Liquid MS supported with filter paper bridge | 4.93$^{b}$ | 3.25$^{b}$ |

Note: Numbers followed by the same letter are not significantly different at DMRT 5%

The use of a liquid medium and high concentrations of endogenous cytokinins causes hyperhydration of the explants (Mazri, 2015) due to the absorption of excess water. Hyperhydration is caused by several factors, such as ammonium levels, the concentration of plant growth regulators and the use of a liquid medium (Mazri, 2015). However, it can be reduced *in vitro* culture by adding solidifying agents, reducing the concentration of cytokinins and nitrogen ions, as well as using buffers in a liquid culture medium (Ivanova and Van Staden, 2011; Grzegorczyk-Karolak et al., 2017).

The shoots from the induction stage were subsequently subcultured on the same medium but without any growth regulators to stimulate their development. Figure 2 demonstrates that the shoot grew well after 8 weeks of culture, as seen in their increased number of shoots and length (Figure 2). The analysis of variance results shown in Table 3 indicated that media type controlled the number of shoots formed and their length. These results are in line with the induction stage. Moreover, the DMRT test also revealed that liquid MS supported with a filter paper bridge produced the highest average number of shoots formed (5.87 shoots explants$^{-1}$) and length (3.64 cm explant$^{-1}$), which were significantly different from those produced by explants cultured on liquid MS supported with viscose sponge and MS medium solidified with 0.25% phytagel as presented in Table 4. The average number of shoots produced which was 5.87 explant, was higher than the results reported in Fauzan et al. (2015b) and Tamyiz et al. (2022), that obtained 1.91 and 1.96 shoots, respectively. This result was lower than the reports in Karlianda et al. (2013) and Akbar et al. (2017), who obtained up to 11 buds explant$^{-1}$.

Table 3. Analysis of variance results of the effect of medium types, NAA concentration and their interaction on agarwood shoot development

| Data | Number of shoots | Shoot length (cm) | Number of leaves |
|---|---|---|---|
| Media type | 6.194* | 4.227* | 1.170 |
| NAA concertation | 0.241 | 1.022 | 0.460 |
| Interaction M x N | 1.501 | 0.854 | 0.898 |

Note: (*) has a significant effect


a. b. c.

Figure 2. The appearance of agarwood shoot, derived from the previous culture at 2 µM NAA, in shoot development state without growth regulators. (a) Shoot grown on liquid MS supported with filter paper bridge; (b) MS medium solidified with 0.25% phytagel; (c) liquid MS supported with viscose sponge

Table 4. DMRT test result on the effect of medium type on the number of shoots and shoot length in media without any growth regulator (n = 15)

| Treatment | Number of shoots | Shoot length (cm) |
|---|---|---|
| Liquid MS supported with viscose sponge | 3.27[a] | 2.65[a] |
| MS medium solidified with 0.25% phytagel | 4.13[a] | 2.93[a] |
| Liquid MS supported with filter paper bridge | 5.87[b] | 3.64[b] |

Note: Numbers followed by the same letter are not significantly different in DMRT 5%

It was also observed that there were two explants, derived from cultured on solid medium supplemented with 6 µM NAA (Figure 3a) and on liquid medium supported with viscose sponge and supplemented with 4 µM NAA (Figure 3b) formed roots, although they were limited in number (1 root explant$^{-1}$) and small in size (0.6 cm). The fact that NAA could not induce root formation contradicted the results of Tamyiz et al. (2022), which states that NAA was the best auxin type to induce *A. malaccensis* rooting.


a. b.

Figure 3. The appearance of agarwood root on media without growth regulator: (a) on an explant derived from culture on solid medium supplemented with 6 µM NAA; and (b) on an explant derived from liquid medium supported with viscose sponge and supplemented with 4 µM NAA

## CONCLUSION

It can be concluded that the development of agarwood shoots (*A. malaccensis*) was controlled by the type of medium used. MS liquid medium supported with a filter paper bridge produced the highest average number of shoots formed (4.93 and 5.87 shoots explants$^{-1}$) and shoots length (3.25 and 3.64 cm explant$^{-1}$) in the induction and development stages, respectively. These results will facilitate the mass propagation of agarwood shoots. However, further study is needed to find the best rooting conditions to produce good agarwood plantlets. The availability of agarwood plantlets will support both conservation and production of *A. malaccensis*.

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