THE APPLICATION OF TWO STEPS CULTURE IN AGARWOOD, *Aquilaria malaccensis*, *IN VITRO* CULTURE IMPROVES MICROSHOOTS INDUCTION AND DEVELOPMENT

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ABSTRACT

Agarwood (*Aquilaria malaccensis* Lamk.) is an important species with high economic value and has many benefits, which led to overexploitation in its natural habitat. An effort to provide sufficient seedlings for both cultivation and conservation of agarwood is therefore needed. This study has been carried out with a view to determine the effect of media types and BAP concentrations in two steps cultivation system on agarwood microshoot induction. This study was a two stage-experiments i.e., microshoot induction and optimizing shoot development. The research results showed that the interaction between different media types and BAP concentrations had no significant effect on agarwood microshoot induction. Subsequent culture on MS medium without any BAP addition showed that explant derived from MS medium solidified with 2.5 g. L^{-1} Phytagel produced 2.36 ± 0.48 shoots/explant and 3.69 ± 1.16 leaves/explant. In addition, explant derived from culture on MS medium supplemented with 4 µM BAP produced 2.28 ± 0.61 shoots/explant. This is for the first time the application of two steps culture system for Agarwood (*A. malaccensis*) has been deployed and how the habituation phenomenon is handled.

KEY WORDS: : *BAP, Multiplication, Murashige & Skoog, Plant Growth Regulator, Types of Media*

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INTRODUCTION

Agarwood (*Aquilaria malaccensis* Lamk.) is a plant that belongs to the *Thymeleaceae* family. Agarwood is an important plant because it has a high economic value. Agarwood is used in religious ceremonies, medicine, perfumes ingredients, and ornamental materials. Those many benefits of agarwood have led to an overexploitation of the agarwood population in nature. Illegal logging and trade of agarwood without sufficient conservation efforts have led to the decline of agarwood population, which is therefore listed as "highly endangered" in appendix II of the Convention on International Trade in Endangered Species (Nath et al., 2020).

Due to the threat to agarwood populations in nature, conservation efforts are needed, such as through mass propagation of agarwood. Agarwood propagation using conventional methods takes a long time and can only produce a limited amount of seedlings. A more efficient method is much needed, such as via *in vitro* culture. Plant *in vitro* culture can produce large quantities of plants in a faster time and free of pests and diseases (George and Sherington 1984).

Plant propagation *via in vitro* culture is influenced by several factors such as the media and plant growth regulators used, as well as the environmental conditions of the culture (George, 1993). The most commonly used medium for plant *in vitro* culture is Murashige and Skoog medium (MS-1962 medium), containing vitamins, carbon source, and inorganic salts (Trivedi et al., 2015).

Media types play important roles in the success of micropropagation. The media type regulates explant osmoregulation, nutrition absorption, and oxygen availability. The types of media that commonly used are solid, semi-solid and liquid media. Solid medium is a nutrient medium with gelling agent as a solidifier. The commonly known solidifiers are agar, agarose, and phytagel, which quite costly and unsuitable for mass production. Liquid medium on the other hands, does not require gelling agent, but a proper explant support system is needed especially for culture other than cell or callus culture. Liquid medium is also recommended to induce rooting (Pierik, 1982).

Microshoot induction is an important step in plant propagation using *in vitro* culture technique. Plant growth regulators (PGR), especially cytokinins, play important roles in inducing shoot formation, improving shoot multiplication, and stimulating cell division. The most commonly used cytokinin in *in vitro* culture is 6-Benzylaminopurine (BAP). BAP has a benzyl ring which making it more stable and more potent compared to other cytokinins such as zeatin and kinetin (George and Sherington, 1984; Ashraf et al., 2014). This study has been carried out with a view to determine the effect of media types and BAP concentrations in two steps cultivation system on agarwood microshoot induction.

METHODS

This study has been conducted from July to October 2021 at the Laboratory of Plant *In Vitro* Culture, Faculty of Biology, Jenderal Soedirman University.

The explant used in this research were shoots which had been continuously sub-cultured on solid Murashige and Skoog medium (MS medium) (Sigma-Aldrich-M519) supplemented with 15 µM of BAP (Sigma-Aldrich-B3408) and incubated at 24 ^{o}C under continuous light.

Microshoot induction has been carried out on MS basal medium (Sigma-Aldrich-M519) supplemented with 20 g.L^{-1} sucrose. This first stage of the study has been carried out experimentally using a Completely Randomized Design (CDR) arranged in a factorial

treatment pattern with three replications. The first factor was the media types consisting of MS medium supplemented with 2.5 g. L^{-1} phytagel, MS liquid medium supported with filter paper bridge, and MS liquid medium supported with viscous sponge. The second factor was the BAP (Sigma-Aldrich-B3408) concentrations at 0 µM, 1 µM, 2 µM, 3 µM, 4 µM. Agarwood explants were inoculated into the treatment media, 1 explant/bottle. The culture was incubated in a culture rack with continuous lighting at a temperature of 24° C for 12 weeks.

This second stage of experiment has been carried out by subculturing the explant from the first step onto MS basal medium supplemented with 20 $g.L^{-1}$ sucrose, with three types of supporting materials (phytagel, filter paper bridge, and sponge), as used in microshoot induction, without any BAP addition. Agarwood explants were sub-cultured into these media, 1 explant/bottle. The culture was incubated on a culture rack with continuous lighting at a temperature of 24° C for 8 weeks.

The variable observed was agarwood microshoot formation, with the parameters measured included the number of shoots, number of leaves, and plant height.

The data obtained were analyzed using an analysis of variance (ANOVA) followed by the Duncan multiple range test with a confidence level of 95%.

RESULT AND DISCUSSION

The analysis of variance (ANOVA) results of the first stage showed that the treatments given did not show any significant difference on agarwood microshoot induction in all measured parameters. Those results indicated that type of media and BAP concentration either sole agent or in combinations are incapable of increasing microshoot formation. However, those results may also show interesting findings, i.e., the habituation and the media type used.

The explants used in this research had been maintained in MS medium supplemented with 15 µM BAP for a substantially long time. In addition, with a view to obtain the same growth phase, the explants were further sub-cultured into solid MS medium supplemented with 5 µM BAP for 12 days, just before being inoculated onto the treatment media. These long exposure to BAP may lead to higher BAP concentration within the explant leading to a habituation phenomenon in culture. BAP is one of PGR which 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 an enzyme called β-Glucosidase to produce free and active BAP (Reinert and Yeoman 1982; Blakesley et al. 1991; Schaller et al. 2014; Feng et al. 2017).

Explant grew on certain media given with the same plant growth regulators continuously, showed habituation in which addition of the same PGR causes no significant change to the explant (Arti and Mukarlina 2017). According to Vardja and Vardja (2001), to maintain explant viability, it is necessary to reduce the accumulation of cytokinin, hence the explants need to be transferred to resting media (MS0).

There are three types of media commonly used in *in vitro* culture, namely solid, semi-solid, and liquid medium (Mbiyu et al. 2012; Alkhateeb and Alturki 2014; Rezali et al. 2017). The medium type affects both the osmolarity of the solution in the medium and the availability of oxygen for explant growth (Basri 2016). Liquid medium is considered a better medium type because it has a high-water potential, which facilitating better water transportation from the medium to the explants (George et al. 2008). The use of explant supporting materials is very important to assure explants getting access to both oxygen and nutrients for their growth (Marlin 2009).

The fact that the type of media used showed no significant difference on all parameters measured indicated that all types of media could be used for microshoot induction of agarwood.

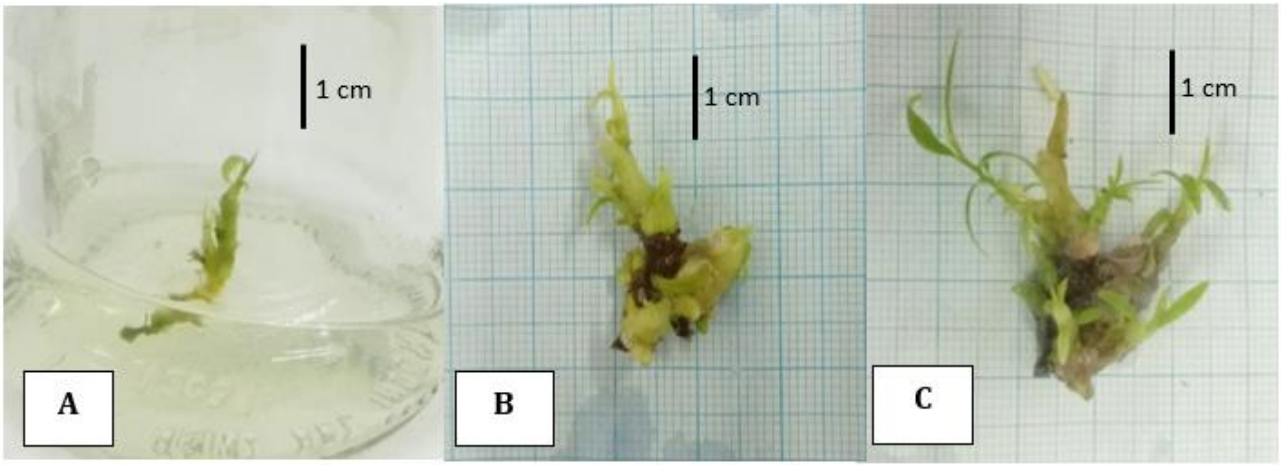

Figure 1. The result of the growth by agarwood explant. (A) Explant culture in the first week of culture in the treatment media. (B) Explant after 12 weeks culture on treatment media. (C) Explant after 8 weeks culture on media withtout BAP

Pierik (1982); Suthar et al., (2011); Ahmadian et al., (2017) also stated that neither solid nor liquid culture media affect the growth of explants in the micropropagation stage of carnation (*Dianthus caryophyllus* L.) and *Boswellia serrata* Roxb., respectively. This finding will be beneficial for mass propagation of agarwood by reducing the cost of solidifying agent.

The second stage of experiment, which was dedicated to optimizing the shoot growth, some significant effects were observed. In this stage, no BAP was applied. According to Vardja and Vardja (2001), this step is necessary to reduce the accumulation of cytokinin. The ANOVA results showed that media type or BAP concentration used in the first stage controlled the number of shoots and leaves formed but did not affect the shoot length. In addition, the interaction between media type and BAP concentrations used in the first stage showed no significant difference effect on all measured parameters in the second stage of experiment.

Visual observation of the explant (Figure 1.) showed the changing of explant from the first week of culture (A), condition after 12 weeks of culture in the treatment media (B), and explant condition after 8 weeks culture on media without BAP. The explant changed both in size, number of shoots and leaves. Figure 1 also showed changes in the leaf colour from light green to much darker one. There is also an increased number of leaves, which both indicate the growth of explant. The Explants also swollen. According to Prasetyo et al. (2020), the swelling of explants indicated that explants had undergone cell division and enlargement, whereas the appearance of shoots and leaves on the explants indicated cell differentiation.

The result of the Duncan multiple range test (Table 1) showed that the best results were obtained from the explants originated from culture on MS medium solidified with phytagel, which produced 2.36 ± 0.48 shoots/explant and 3.69 ± 1.16 leaves/explant (Figure 2). Those results indicated that solid MS medium (MS + Phytagel) is more effective in supporting agarwood explant growth. This finding was also consistent with the finding of (Sembiring et al. 2018) who reported that the use of solid media on orchid *Cattleya trianae* Lindl & Rchb.fil. resulted in a better percentage of shoot formation (100%) compared to liquid media (89.75%). The possible reason why liquid media not suitable for aforementioned explant, might be due to the incident of vitrification.

Figure 1. part C shows the characteristics of vitrification, as shown in colourless shoot. Vitrification is an increased water content in explants also called waterlogging. Vitrification occurs probably due to the high potassium (K) content. The high cytokinin content in explants might cause high K content.

Table 1. The effect of previous media types on subsequent explant growth on media without BAP

Media Types	Number of shoots	Number of leaves
MS + Phytagel	2.36 ± 0.48[a]	3.69 ± 1.16[a]
MS + Filter paper bridge	2.09 ± 0.65[ab]	2.82 ± 0.72[b]
MS + Viscous Sponge	1.81 ± 0.51[b]	2.59 ± 1.08[b]

Details: The number followed by the letter "ns" shows the results are not significantly different and numbers followed by the same letter are not significantly different in DMRT 5%

Cytokinin and K content are co-regulated due to the similarity of the receptor, namely HAK5 (Vardja and Vardja, 2001; Nam et al., 2012). Potassium regulates water transport in plants. It does so by maintaining water retention inside plant cells, maintaining cell turgor, and maintaining low transpiration rate (Wang et al. 2013). Since the explant is grown *in vitro*, the transpiration rate goes even slower. This combination of events leads to vitrification of explant. Vitrification can be overcome by reducing explant contact to water. The use of phytagel as a solidifying agent play an important role in reducing the incidence of vitrification leading to better shoot growth.

The explant was then sub-cultured into media without BAP to avoid vitrification due to BAP accumulation. This sub-culturing was effective in inducing microshoots of agarwood. According to Hafizh et al. (2018), culturing *Fragaria chiloensis* on MS medium without any addition of cytokinin, resulted in the increase of shoot diameters and explant growth.

The result of the Duncan multiple range test on the effect of previous BAP concentration on subsequent growth of explant on media without BAP (Table 2) showed that explant derived from 4 µM BAP culture produced highest number of shoots 2.28 ± 0.61 shoots/explant, which was significantly different with explant derived from media without BAP. However, the number of shoots produced under 4 µM BAP was not significantly different with those of produced by explant derived from 1 µM, 2 µM, 3 µM BAP treatments.

Table 2. The effect of previous BAP concentrations on subsequent explant growth on media without BAP

Concentrations of BAP	Number of shoots
0 µM	1.52 ± 0.37[b]
1 µM	2.22 ± 0.57[a]
2 µM	2.20 ± 0.62[a]
3 µM	2.22 ± 0.44[a]
4 µM	2.28 ± 0.61[a]

Details: The number followed by the letter "ns" shows the results are not significantly different and numbers followed by the same letter are not significantly different in DMRT 5%

BAP is a cytokinin which has a benzyl ring which makes it more stable and more potent compared to other cytokinins such as zeatin and kinetin. BAP plays an important role in stimulating cell division and chloroplast maturations leading to cell regeneration, as well as inducing shoot formation and multiplication (George and Sherington, 1984; Ashraf et al., 2014).

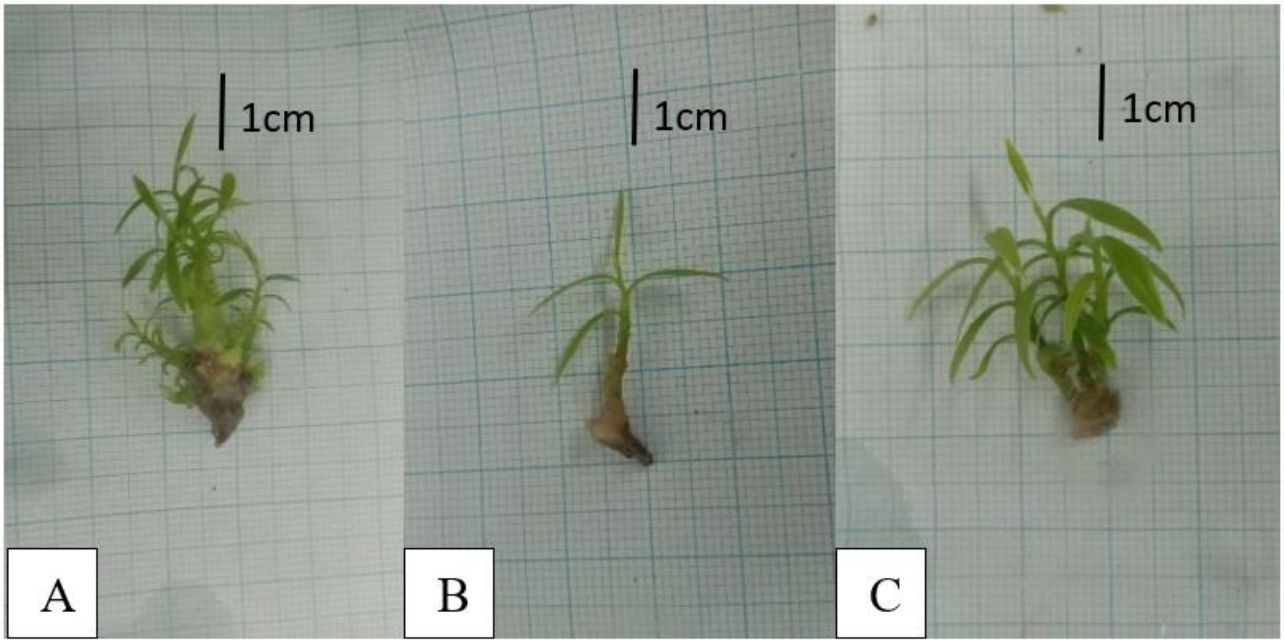

Figure 2. The appearance of microshoots induction of agarwood. (A) Agarwood on MS + phytagel + 1 µM. (B) Agarwood on MS + Viscous Sponge + 1 µM. (C) Agarwood on MS + Viscous Sponge + 3 µM

Those results indicated that transferring the explant into media without BAP could reduce accumulation of BAP. According to Karyadi and Ahmad (2008) axillary bud formation can be stimulated by transferring explant into MS medium without PGR. The best BAP concentration found was 4 µM, which was higher than that of reported by (Wardatutthoyyibah et al., 2015). Wardatutthoyyibah et al. (2015) reported that the addition of 0.5 mg. L^{-1} BAP (1 mg. L^{-1} is equivalent to 4.4 µM) resulted in the best agarwood (*A. malaccensis*) shoot formation, which produced up to 12 shoots/explant.

This low number of shoots produced could be caused by high BAP content due to BAP pre-treatment and periodical sub-culture in BAP containing media, since BAP is easily conjugated into the explant. Kamínek et al. (1997); Maxiselly et al. (2020), showed that the addition of BAP causes plants to have cytokinin accumulation and increase the cytokinin ratio in plants. Addition of exogenous cytokinin (BAP) can induce habituation for explants. According to Vardja and Vardja (2001), continuously subculturing the explant at the same media contain cytokinin resulted in a decrease of explant growth at the 7th subculture.

CONCLUSION

It can be concluded that two-steps culture improved microshoot induction and development. After 8 weeks cultured on MS medium without BAP, explant derived from MS medium solidified with 2.5 g. L^{-1} Phytagel produced 2.36 ± 0.48 shoots/explant and 3.69 ± 1.16 leaves/explant. Moreover, explant derived from MS medium supplemented with 4 µM BAP produced 2.28 ± 0.61 shoots/explant when cultured on MS medium without BAP for 8 weeks.

ACKNOWLEDGEMENT

The authors wish to thank to Dean and Vice Dean for Academic Affairs Faculty of Biology, Jenderal Soedirman University for the permission given to carry out this study.

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