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SHOOT MULTIPLICATION AND GROWTH RATES OF *AQUILARIA MALACCENSIS* LAMK. SHOOT CULTURES IN TEMPORARY IMMERSION SYSTEM (TIS)-RITA® AND BUBBLE COLUMN BIOREACTORS

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Abstract

Agarwood is the resinous product of several tree species of family Thymelaeaceae such as *Aquilaria* spp. Unsustainable harvesting has threatened the existence of agarwood producing species in their natural habitat. Therefore, the present study was developed to facilitate an effective and efficient method to produce *Aquilaria malaccensis* seedlings by *In vitro* culture. Here, we optimized the aeration rate in the bubble column reactor and the immersion time on the temporary immersion system (TIS)-RITA® bioreactor and compare the growth rate of *A. malaccensis* with both systems. *A. malaccensis* shoot cultures were propagated in Murashige and Skoog (MS) semisolid medium and then pre-conditioned in thin layer culture before bioreactor cultivation. *A. malaccensis* shoots in the bubble column reactor were subjected to variable aeration rates of 0.05 and 0.1 vvm, while the immersion variation within the TIS-RITA® bioreactor was 5 and 15 min for every 4 h. The results showed that 15 min immersion for every 4 h increased the number of *A. malaccensis* shoot regeneration. Nonetheless, no significance difference was observed on biomass acquisition in both bubble column bioreactor and TIS-RITA® bioreactor. Therefore, our findings indicate that immersion period was more critical than aeration rate for *A. malaccensis* shoot cultivation.

Key words: *Aquilaria malaccensis*, Bubble column bioreactor, Shoot multiplication, TIS-RITA® bioreactor.

Introduction

Agarwood is the trade name of non-wood products produced by some tree species of the genera *Aquilaria* and *Gyrinops*. It is a part of the wood that forms resins containing aromatic secondary metabolites (Faizal *et al.*, 2017; Kalra & Kaushik, 2017). The strong aroma is a complex mix compounds, hence its oil could be used as a fragrance, whereas its powder is used for incense and medicinal purposes (Naef, 2011).

Indonesia is one of the largest agarwood producers in the world. In 1990, Indonesia exported 600 tons per year of agarwood, but production began to decline in the 2000s (Sitepu *et al.*, 2011; Turjaman & Hidayat, 2017). The high price agarwood has driven producers to change their method. While the previously harvested from trees that had died naturally, they now cut living trees. This has threatened the sustainability of natural agarwood resources. Therefore since 2004, two genera of agarwood-producing trees were listed as endangered plants in Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) (CITES, 2004).

In addition to excessive exploitation, the number of agarwood-producing trees is hampered by the limited production of agarwood seedlings. Conventionally, agarwood seedlings is propagated through generative or vegetative reproduction method, but both techniques require long periods of time and have relatively low successful rates. *Aquilaria* trees only start to flower and produce fruit after 5-6 years (Sitepu *et al.*, 2011), and agarwood seeds are recalcitrant with low viability (Kundu & Kachari, 2000; Kundu & Sett, 2005). Therefore, *In vitro* propagation offers an effective and efficient solution to produce agarwood seedlings. This technology could facilitate the production of large number of seedling in a relatively short time period (Meng-Ling *et al.*, 2005). The current study reports for the first time on the effects of a bubble column reactor and a temporary immersion system, (the Recipient for Automated Temporary Immersion System TIS-RITA®) on biomass, shoot growth and shoot multiplication in an agarwood-producing plant (*Aquilaria malaccensis*).

Materials and Methods

Propagation of *A. malaccensis* shoot culture in semisolid medium: The sterile shoot cultures were subcultured onto semisolid Murashige-Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with 30 g/L sucrose, 8 g/L agar, and 0.5 and 1 mg/L 6-benzylaminopurine (BAP). The pH medium level was maintained in the range of 5.6 to 5.8. Shoot cultures were incubated in a controlled environment with a light intensity of 700 lux with a 12-/12-h light/dark photoperiod at room temperature.

Pre-conditioning of *A. malaccensis* shoot culture to liquid medium by thin layer culture: Shoot cultures in semisolid medium longer than 2 cm were transferred into Erlenmeyer flasks containing 5 mL half-strength liquid MS medium and 0.5 mg/L BAP. *A. malaccensis* shoot cultures were incubated for 7 days on a rotary shaker (60 rpm) at the same conditions as mentioned in semisolid culture.

***A. malaccensis* shoot cultivation in the bubble column reactor:** *A. malaccensis* shoots (2.5 g fresh weight) were fed into a hand-made bubble column reactor (4.5 cm diameter, 35 cm height, and maximum capacity 500 mL) with 250 mL of half-strength liquid MS medium

supplemented with 30 g/L sucrose and 0.5 mg/L BAP. Agarwood shoots were cultivated with different aeration rates of 0.1 vvm and 0.05 vvm and incubated for 21 days (Kusakari *et al.*, 2012).

***A. malaccensis* shoot cultivation in the TIS-RITA® bioreactor:** The 1-litre TIS-RITA® bioreactor comprises two compartments, 6.5 cm diameter for an upper vessel with the plant material and 7.5 cm diameter for a lower one with the medium. The *A. malaccensis* shoot culture incorporated into the TIS-RITA® bioreactor was approximately 4 g fresh weight. A 250 mL volume of the same medium employed in the bubble column was also used in the TIS-RITA® bioreactor. The immersion time was adjusted to 15 min per 4 h according to the description by Malosso *et al.*, (2012) and Debnath (2009) related to *Jacaranda decurrens* and *Vaccinium angustifolium* shoot cultures, respectively. The variation of the immersion time 5 min/4 h was based on research related to the effects of hyperhydricity that inhibit increase of plant biomass (Snyman *et al.*, 2011). Therefore, immersion times for 21-days cultivation were 5 and 15 min every 4 h, respectively.

Shoot measurement: *A. malaccensis* shoot growth was evaluated by weighing the initial and final fresh weights using an analytical scale. The dry agarwood shoots were then cut into small pieces and put into a sealed container for freeze drying for 8 h. The height of agarwood shoots was measured by using a millimeter block, and the number of shoots was counted manually.

Root initiation: Multiple *A. malaccensis* shoots formed through shoot induction in both bioreactors were then isolated and transferred into liquid MS media supplemented with 1 mg/L indole-3-butyric acid (IBA) and 0.01 mg/L indole-3-acetic acid (IAA) for 8 weeks. Shoot cultures were incubated in a controlled environment with a light intensity of 700 lux with a 12-/12-h light/dark photoperiod at room temperature.

Medium analysis: The conductivity of medium samples at baseline ($t = 0$) and after cultivation ($t = t_t$) was measured using a conductivity meter (LT Lutron CD-4303), and sucrose content was measured with a refractometer (Milwaukee MA871).

Statistical analysis: Statistical analysis was conducted using the Kruskal-Wallis test with significance level set at $p<0.05$. Each bioreactor was considered as one replicate. The presented experiments are comprised of at least three replicates.

Results and Discussion

Initiation and acclimatization: *A. malaccensis* shoot culture growth was optimized with MS semisolid medium and the cytokinin BAP at concentrations of 0.5 and 1 mg/L. Observations were made for 4 weeks under the same environmental conditions. The final shoot culture appearance is shown in Figure 1. Based on visual observation, tubers and stems in the control shoots (without hormone) grew tall, and had normal stem and tuber morphology with no enlargement (Fig. 1B). The stumps and stems of explants with 0.5 and 1 ppm BAP grew slightly enlarged, but the leaves were still in normal condition.

Research into multiplication of *A. hirta* shoots conducted by Hassan *et al.*, (2011) using a concentration of 0.5 mg/L BAP yielded 4.9 shoots/explant, while 1 mg/L yielded 4.1 shoots/culture. Azwin *et al*., (2006) reported that *A. malaccensis* shoots cultured with 0.5 and 1 mg/L BAP yielded 5.67 shoots/explant and 4.44 shoots/explant, respectively. In the present study, propagation of shoots without growth regulator yielded 3.67 shoots/explant, and the addition of 0.5 and 1 mg/L BAP produced 6.67 and 3.67 shoots/explant, respectively. Based on morphology and shoot height after 4 weeks, 0.5 mg/L BAP was used for shoot growth and multiplication in subsequent subcultures and bioreactor experiment.

Prior to bioreactor cultivation, explants were pre-conditioned in thin layer culture (TLC) using half-strength MS without hormones. Oberschelp & Gonçalves (2016) found that decreasing the salt content in MS medium could increase the number of shoots and roots, so this approach was adopted. Pre-conditioning was carried out to adapt the explants in liquid medium and subsequently allowing the optimal plant response upon cultured in bioreactors. The initial and final appearance of explants after 3-7 days of pre-condition are shown in Figure 2.

Based on visual observations, pre-conditioning produced a healthy stems and normal leaves that were not opaque and not easily detachable. These explants were still fairly normal in terms of morphology, and could be used further for treatment in bioreactor. In the TLC system, shoot cultures did not multiply, but they obtain a greater height increase (Fig. 2).

Bioreactor culture of *A. malaccensis* shoots: Explant treated with 0.5 and 1 mg/L BAP did not show symptoms of hyperhidricity (Fig. 3). Hyperhydric explants tend to be translucent with leaves that are easily rolled and detached (Rojas-Martínez *et al.*, 2010; Gao *et al.*, 2018). Visually, shoots and growth in both systems were normal; the shoots were green, not opaque, did not show stem enlargement, and had normal leaf growth. Figure 3 shows *A. malaccensis* shoot cultures after 3 weeks in the bioreactors.

Different initial fresh weight was used as both bioreactors have different specifications. Furthermore, the initial fresh weigh was adapted from our preliminary experiment on these two reactors (data not shown). Table 1 lists the increase on biomass acquisition for the two bioreactor systems. In TIS-RITA®, the immersion treatments of 15 min resulted in higher biomass increases of about 1.8 fold. The increase in the fresh shoot weight for 5 min and 15 min immersions was 1.52 ± 0.56 and 3.12 ± 0.27 g, respectively. In the bubble column, the increase in fresh weight was not significantly different (both ~ 1.8 fold).

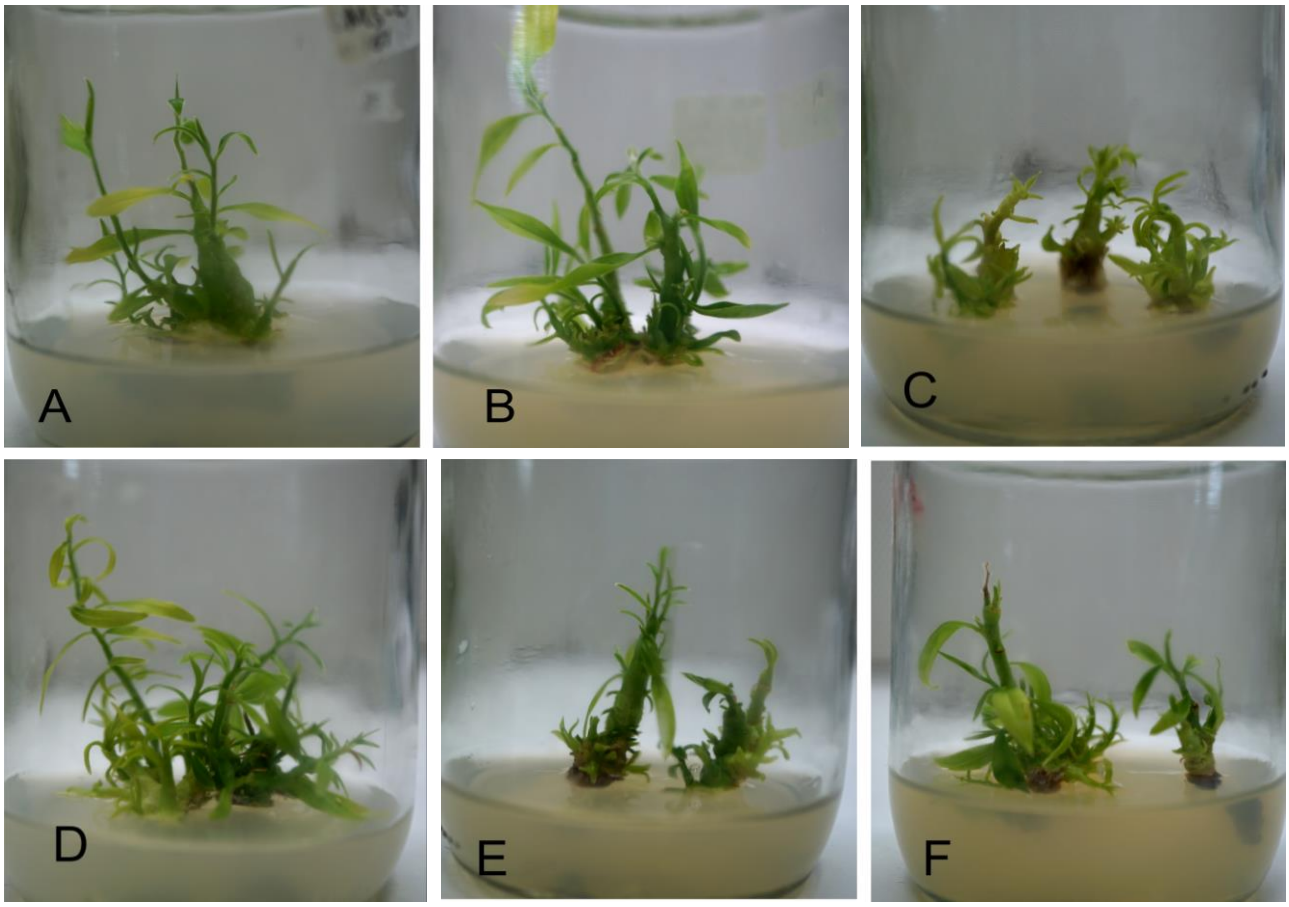

Fig. 1. Explant appearance of *A. malaccensis* shoots on solid medium grown under different conditions. (A) Without growth regulator for 2 weeks, (B) without growth regulator for 4 weeks, (C) BAP 0.5 for 2 weeks, (D) BAP 0.5 for 4 weeks, (E) BAP 1 ppm for 2 weeks, and (F) BAP 1 ppm for 4 weeks.

Number of shoot was significantly different between treatments in the TIS-RITA® bioreactor (3.00 and 5.12 shoots/initial explant for 5 min and 15 min, respectively; Table 1). On the other hand, no significant difference was observed on shoot length after 21 days in culture. Likewise, shoot length in the bubble column bioreactor was not significantly different for both treatments, despite they produced lower number of shoots compared to the TIS-RITA® bioreactor. Therefore, time of exposure to the medium was more critical for increasing number of shoot multiplication rather than the total biomass.

The amount of sucrose in the bioreactor medium decreased with culturing (Table 1). Sucrose is an essential factor for plant biomass growth in *In vitro* system. It is the carbon source for plant cells, acting as a source of energy for growth, biosynthesis, and other metabolic processes (Satish *et al.*, 2016). Therefore, the decrease in sucrose level is proportional to shoot multiplication.

Immersion is thought to affect the internal gas conditions in the medium environment of shoot cultures, which reduce the concentration of O_2 and increase the concentrations of CO_2 and ethylene in the bioreactor (Kiferle *et al.*, 2014; Regueira *et al.*, 2018). Higher ethylene gas levels affect the decrease of abscisic acid concentration in culture. This can lead to increased gibberellin response in shoot cultures, resulting in greater shoot growth (Regueira *et al.*, 2018). Contact between the medium and explant was continuous in the bubble column bioreactor and prolonged contact between the BAP and shoots would induce greater shoot multiplication compared to that in the TIS-RITA® bioreactor. BAP is a synthetic growth regulator of the cytokinin class, with impact in shoot multiplication (Mok & Mok, 2001; Faizal *et al.*, 2011).

The medium conductivity was measured at the end culture period to check ionic content by comparing with initial value of 5.7 S/m (Table 1). The shoots were cultivated in medium containing macro- and micronutrient ions to facilitate shoot growth. The decline in conductivity showed that ions were absorbed by explants and used for growth (Jang *et al.*, 2016, Sønsteby *et al.*, 2017). The aeration system causes a partial pressure change of CO_2; thus, CO_2 is dissolved in a liquid medium. Dissolved CO_2 in water reacts with water molecules (H_2O) to form hydrogen carbonate (H_2CO_3), which then dissociates into HCO_3^- and hydrogen ions (H^+) (Welander *et al.*, 2014). This may cause increase medium conductivity in the bioreactor. Subsequently, the growth of the culture resulted in uptake of nutrients from the medium thereby reducing the conductivity of the medium. However, this could not explain the smaller decrease in the conductivity in bubble column compared to the TIS-RITA® bioreactor as we did not have an estimation of real amount of dissolved CO_2 in both systems.

Most species require auxin treatment for root induction prior to acclimatization. Similarly, *A. malaccensis* regenerated shoots in the two bioreactor systems developed roots after being cultured in liquid MS media supplemented with 1 mg/L indole-3-butyric acid (IBA) and 0.01 mg/L indole-3-acetic acid (IAA) for 8 weeks with an efficiency of 100% (Fig. 4).

Table 1. Effect of the type of bioreactor on the shoot regeneration; shoot length, biomass acquisition, medium conductivity, and sucrose consumption.

Bioreactor	Treatment	Number of shoot	Length of shoot (cm)	Δ Fresh weight (g)	Δ conductivity (S/m)	Sucrose consumption (g)
TIS-RITA®	5 min	3.00 ± 0.42^{b}	2.68 ± 0.22	1.52 ± 0.56	0.76 ± 0.69^{a}	1.79 ± 0.66^{b}
	15 min	5.12 ± 0.48^{a}	2.60 ± 0.28	3.12 ± 0.27	0.58 ± 0.81^{a}	1.96 ± 0.25^{a}
Bubble Column	0.05 vvm	2.14 ± 0.69^{b}	2.38 ± 0.59	1.26 ± 0.31	0.12 ± 0.07^{b}	1.35 ± 0.13^{b}
	0.1 vvm	2.12 ± 0.83^{b}	2.23 ± 0.65	1.66 ± 0.16	0.08 ± 0.05^{b}	1.65 ± 0.23^{b}

Δ Fresh weight = final weight – initial weight for each bioreactor; Δ conductivity = final conductivity – initial conductivity (5.7 S/m); Sucrose consumption: final sucrose (g) – initial sucrose (g)
Different letters indicate significant difference (Kruskal-Wallis $p<0.05$)

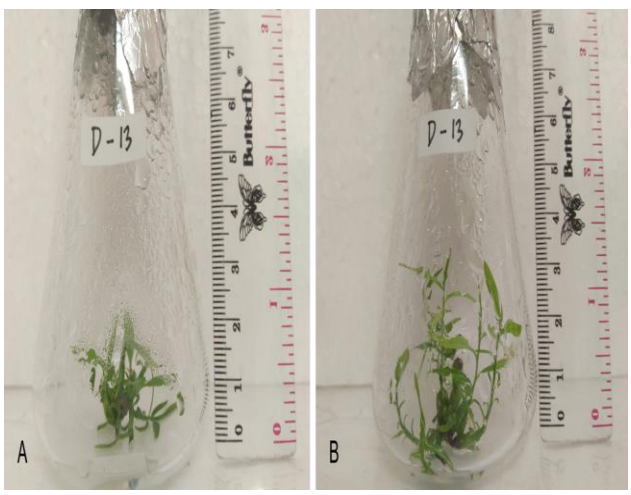

Fig. 2. Shoots of *A. malaccensis* before (A) and after (B) pre-conditioning to liquid medium culture by a TLC system.

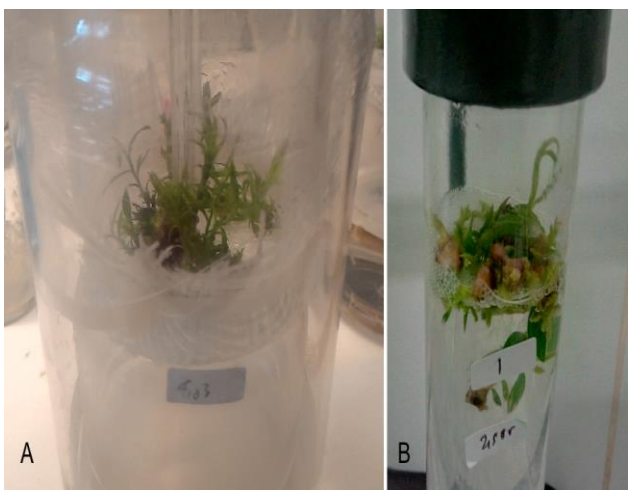

Fig. 3. *A. malaccensis* culture conditions in the TIS-RITA® bioreactor (A) and in the bubble column after 3 weeks of treatment.

Fig. 4. *In vitro* rooting of regenerated *A. malaccensis* shoot culture in liquid MS medium.

Conclusion

Longer immersion duration positively impacted *A. malaccensis* shoot multiplication in the TIS-RITA® bioreactor. 15 min immersion resulted in significant increase in shoot regeneration (5.12 shoots/initial explant) compared to 5 min immersion for every 4 h (3.00 shoots/initial explant). In contrast, different aeration rate using bubble column bioreactor was not sufficient to induce higher shoot regeneration in *A. malaccensis*.

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