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Agarwood (*Aquilaria malaccensis*) diversity conservation by in vitro culture with IAA and yeast extract

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Abstract. *Samanhudi, Sakya AT, Setyawati A, Muawanah M. 2022. Agarwood (*Aquilaria malaccensis*) diversity conservation by in vitro culture with IAA and yeast extract. Biodiversitas 23: 2457-2463.* Agarwood (*Aquilaria malaccensis*) is a scare plant with high economic value and many benefits. Tissue culture is an effective way of producing plants in large quantities, in a short time with uniform results. This study aims to get the proper concentration of IAA and yeast extract for agarwood growth in vitro. The research was conducted in December 2019-June 2020 at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta. The design used is Randomized Complete Block Design (RCBD) in 2 factors: first factor was IAA concentration with 4 level: 0; 0,5; 1; 1.5 mgL^{-1} and second factor was yeast extract concentration with 4 levels: 0; 200; 400; 800 mgL^{-1}. Obtained 16 combinations of treatments repeated 3 times so that there are 48 units of experiments. Observed variables: the time of appearance of shoots, the number of shoots, the height of shoots, the number of leaves, and the number of roots. Root count data is described and data other than root count is analyzed ANOVA, if real influence is further tested DMRT 5%. The IAA affects the height of shoots and the number of agarwood leaves in vitro rooted plantlets has 5 plantlets.

Keywords: *Aquilaria malaccensis*, gaharu, IAA, in vitro, yeast extract

INTRODUCTION

Agarwood usually called gaharu in Indonesia (*Aquilaria malaccensis*) is an annual plant that produces a distinctive aroma with multifunctional benefits, such as health, beauty industry, and even religious rituals. As a superior plant that has high value (Lee et al. 2016), gaharu is much sought after and included in Appendix II (scare) list due to the high market demand from nature in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Dwianto et al. 2019). Several sources state that agarwood is difficult to find in its natural habitat (Suharti et al. 2011; Tan et al. 2019), it will produce seeds after 7-9 years (Ali and Kashem 2019). Therefore, conservation is needed in order to preserve the diversity of agarwood.

Propagation of agarwood plants with tissue culture techniques is an effective way to produce large quantities of plants in a short time with uniform results (Baday 2018). Tissue culture has been significant role in producing disease-free planting materials (Tegen and Mohammed 2016) and can produce many seeds quality (Salam et al. 2019). Multiplication of agarwood through in vitro techniques can support the mass propagation of seedlings (Fauzan et al. 2015). Sterilization methods can affect plantlet growth (Widiastuti et al. 2018). Tissue culture with substances with proper growth regulators and accompanied by optimal light can produce callus regeneration and shoots (Chen et al. 2019).

Growth regulatory substances in tissue culture serve to help accelerate growth and development. Auxin includes growth regulators that can help the process of lateral root formation (Alarcón et al. 2019). Indole acetic acid is a type of auxin that is often used in tissue culture. Yuniastuti et al. (2018) research show WPM media added 4 ppm BAP and 1 ppm IAA produces the best growth in buds and leaves of *Sterculia foetida*.

Yeast is the result of fermentation that can be used as a mixture/substitute for one of the media materials of tissue culture. Yeast extract contains free alpha-amino nitrogen, minerals and vitamins (Jacob et al. 2019). Hassan et al. (2018) research state that root in vitro with yeast extract of 100 mgL^{-1} for 1 day can obtain a high sesquiterpene compound. This study aims to get the right concentration of IAA and yeast extract for agarwood growth in vitro.

MATERIALS AND METHODS

Study period and area

This research was conducted from December 2019 to June 2020 in the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Indonesia.

Field experimental procedure

The implementation of research includes sterilization of tools, media making, planting plantlets, maintenance and

observation of planlets. This study used a Complete Randomized Group Design (CRGD) factorial which is used 2 factors, first factor is concentration of IAA (Indole Acetic Acid) with the level concentration by 0 mgL^{-1} (A0); 0.5 mgL^{-1} (A1); 1 mgL^{-1} (A2); 1.5 mgL^{-1} (A3) and second factor is yeast extract with the level concentration by 0 mgL^{-1} (R0); 200 mgL^{-1} (R1); 400 mgL^{-1} (R2); 800 mgL^{-1} (R3). The agarwood planlet needed in the study amounted to 22 subcultured planlets into 160 planlets (16 combinations of treatment repeated 10 times), then taken 48 best planlets (each combination there are 3 repeats) treatment after completion of the study (20 Weeks After Planting). Variable time of appearance of shoots is observed every day. Variable observations of the number of shoots, the height of the shoots, the number of leaves, and the number of roots are carried out each week. The overall condition of the planlet (160 planlets) is a healthy planlet, a planlet contaminated with bacteria or fungi, and a dead planlet.

Tools sterilization

The sterilization of a product is intended to obtain a sterile product. dissection tools such as tweezers, culture knife (scalpel), and petri dish are washed to clean. Then put in an autoclave for sterilization with a temperature of 121ºC, a pressure of 1 atm for 30 minutes then stored in the oven to maintain the sterility of the appliance.

Media

Making the media begins by mixing several ingredients such as sugar by 30 g, MS macronutrients 50 mL, MS micronutrients by 10 mL, Fe-EDTA by 50 mL, vitamins by 50 mL, IAA (0 mgL^{-1} (A0); 0.5 mgL^{-1} (A1); 1 mgL^{-1} (A2); 1.5 mgL^{-1} (A3)) and yeast extract (0 mgL^{-1} (R0); 200 mgL^{-1} (R1); 400 mgL^{-1} (R2); 800 mgL^{-1} (R3)), then add the distilled water to a volume of 1000 mL and controlling the pH (about 6.0-6.2). As the controller, NaOH is used for decreasing and HCl is used for increasing pH. Furthermore, add 8 g of agar powder then heat by magnetic stirrer to boil and put it into culture bottle then autoclaved.

Yeast extract

The organic material used is yeast extract which is containing yeast extract, peptone, and agar. Yeast was weighed according to each treatment (0 mgL^{-1}; 200 mgL^{-1}; 400 mgL^{-1}; 800 mgL^{-1}).

Plant cultivation

The samples were obtained from laboratory of Southeast Asian Regional Centre for Tropical Biology (BIOTROP), Bogor, West Java as a plantlet with 0 MS medium (without hormone) (Figure 1). This research was conducted by transferring agarwood plantlet from the initial medium to the treatment medium that the shoot as sub-cultured. Firstly, it was separating the agarwood plantlets from the initial media, then cutting the agarwood plantlets into several shoots about 1 cm in length. Furthermore, the plantlets are cleaned from the roots and leave 2 leaves. The clean plantlets were planted on the treatment medium using tweezers.

Data analysis

All data from the observation were analyzed using ANOVA analysis except for the number of roots, if there was a significant effect, a 5% DMRT (Duncan Multiple Range Test) continued test was carried out. The data from the observation of the number of roots were described.

RESULTS AND DISCUSSION

Shoot appearance time

The criteria of shoots that it is calculated when the shoot has reached a length of 0.5 cm (Figure 2). New shoot planlet reaches 0.5 cm at different times, because the ability of each planlet in absorbing nutrients varies, so it is necessary to observe the time of appearance of shoots. The results of ANOVA analysis showed that each treatment of IAA and yeast extract has no significant effect, as well as the interaction of IAA and yeast extract which has no significant effect on the time of appearance of agarwood plantlet shoots. It is suspected that the concentration of IAA and yeast extract is less effective against the time of appearance of agarwood shoots. The use of plant growth regulators can affect plant growth (Ulfa et al. 2013).

Figure 1. Agarwood plantlet as a parental for subculture

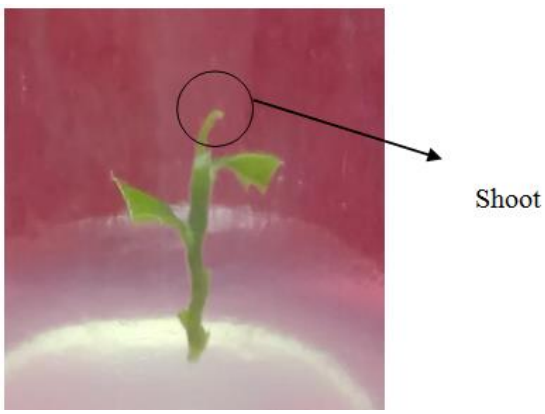

Figure 2. The appearance of shoots on agarwood plantlet

Figure 3 shows that the combination of IAA treatment by 1 mgL^{-1} and yeast extract by 800 mgL^{-1} have the fastest average bud appearance time among other treatment combinations of 15 DAP (Days After Plant). Chhalgri et al. (2020) research suggests that media MS containing 3 mgL^{-1}, 3 mgL^{-1} IAA, and 30 gL^{-1} sugar produced the fastest shoot initiation on rose. Arhvitasari et al. (2019) reveal that a combination of cytokinin 0,8 ppm and auxins 0,1 ppm could accelerate the formation of shoots. IAA by 1.5 mgL^{-1} and yeast extract by 200 mgL^{-1} are treatments with the longest average shoot appearance time of 61 DAP. Avila-Treviño et al. (2017) research that 1 mgL^{-1} BAP + 0.2 mgL^{-1} IAA is the best regeneration with *Moringa oleifera* stem explant.

The concentration of IAA 1.5 mgL^{-1} and yeast extract 200 mgL^{-1} are resulting in the longest average shoot appearance time by 61 DAP (Figure 3). Incompatibility of the IAA and yeast extracts concentrations could slow down the process of the appearance of shoots on agarwood. Sitinjak et al. (2015) declare that the compatibility of the growth regulatory plant substances concentration can be affected plant growth. Chamandoosti (2017) states that the interaction between growth regulatory plant substances greatly affects tissue culture. Moreover, IAA in high concentrations can prolong the appearance of shoots by suppressing the growth of the number of shoots (Lathyfah and Endah 2016).

Number of shoots

According to the analysis result that the concentrations of IAA and yeast extracts were not significantly affected the shoot's number of agarwood. Wardatutthoyyibah et al. (2015) declared that the treatment without auxins can increase the number of shoots that it was not significantly different from the treatment using auxins. Figure 4 shows that the combination of IAA 1 mgL^{-1} and yeast extract 200 mgL^{-1} (A2R1) have the highest average number of shoots among other treatments by 2.33. Media MS containing 3 mgL^{-1}, 3 mgL^{-1} IAA, and 30 gL^{-1} sugar is producing the highest number (3,55) of shoot rose (Chhalgri et al. 2020). In the case of KIN and IAA combinations, the maximum shoot bud induction rate (69.25%) with 6.43 shoots/explant, was noticed on MS medium supplemented with 4.0 mg/l KIN and 0.5 mg/L IAA (Venkatachalam et al. 2015). The number of seaweed shoots was highest (10,6) in Grund media with IAA+BAP (1:1) treatment, and 6,82 with IAA treatment in PES media (Fadel et al. 2013). For direct organogenesis (from axillary bud shoot clumps), 0.1 mg/L IAA and 1.5 mg/L TDZ were found to be optimal for shoot regeneration of stem tips, with mean numbers of axillary bud shoot clumps 7.12±1.23 were produced (Zou et al. 2019).

The use of yeast extract in the media may support germination of orchid seeds, supports the formation of seedlings, produces a strong planlet, as well as effective to increase the number of protocorm-like bodies (PLB), induces the formation of buds, stimulates growth and development of planlets under the greenhouse (Utami and Hariyanto 2020). Hucker et al. (2016) declared that yeast contains thiamin which is the use of thiamine foliar at concentrations of 250, 500, 750 ppm could support the growth and development of coriander and fenugreek by increasing phenolic chlorophyll, carotenoids and antioxidant compounds (Aminifard et al. 2018). Yeast contains thiamin (Hucker et al. 2016). The use of thiamine foliar at concentrations of 250, 500, 750 ppm can support the growth and development of coriander and fenugreek by increasing phenolic chlorophyll, carotenoids and antioxidant compounds (Aminifard et al. 2018).

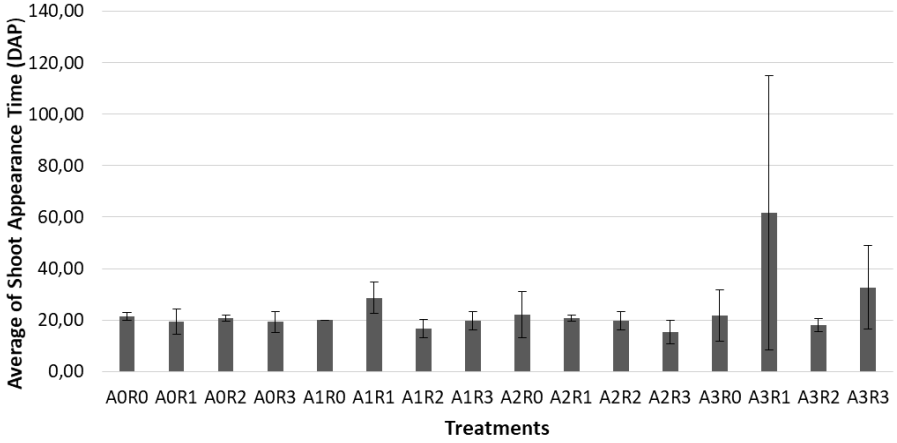

Figure 3. Average shoot appearance time for IAA and yeast extract

A0R0 : IAA 0 mgL^{-1} and yeast extract 0 mgL^{-1}
A0R1 : IAA 0 mgL^{-1} and yeast extract 200 mgL^{-1}
A0R2 : IAA 0 mgL^{-1} and yeast extract 400 mgL^{-1}
A0R3 : IAA 0 mgL^{-1} and yeast extract 800 mgL^{-1}
A1R0 : IAA 0.5 mgL^{-1} and yeast extract 0 mgL^{-1}
A1R1 : IAA 0.5 mgL^{-1} and yeast extract 200 mgL^{-1}
A1R2 : IAA 0.5 mgL^{-1} and yeast extract 400 mgL^{-1}
A1R3 : IAA 0.5 mgL^{-1} and yeast extract 800 mgL^{-1}
A2R0 : IAA 1.0 mgL^{-1} and yeast extract 0 mgL^{-1}
A2R1 : IAA 1.0 mgL^{-1} and yeast extract 200 mgL^{-1}
A2R2 : IAA 1.0 mgL^{-1} and yeast extract 400 mgL^{-1}
A2R3 : IAA 1.0 mgL^{-1} and yeast extract 800 mgL^{-1}
A3R0 : IAA 1.5 mgL^{-1} and yeast extract 0 mgL^{-1}
A3R1 : IAA 1.5 mgL^{-1} and yeast extract 200 mgL^{-1}
A3R2 : IAA 1.5 mgL^{-1} and yeast extract 400 mgL^{-1}
A3R3: IAA 1.5 mgL^{-1} and yeast extract 800 mgL^{-1}

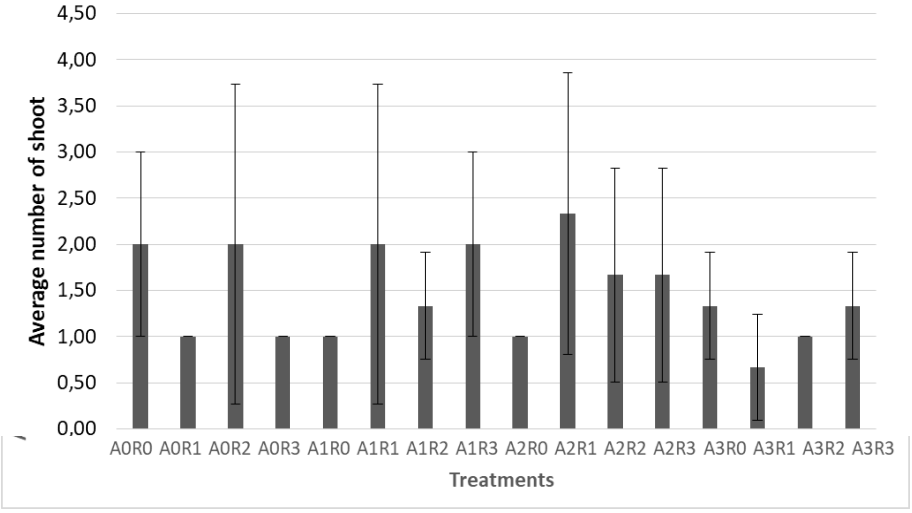

Figure 4. The average of the shoot number for IAA and yeast extract

A0R0 : IAA 0 mgL^{-1} and yeast extract 0 mgL^{-1}
A0R1 : IAA 0 mgL^{-1} and yeast extract 200 mgL^{-1}
A0R2 : IAA 0 mgL^{-1} and yeast extract 400 mgL^{-1}
A0R3 : IAA 0 mgL^{-1} and yeast extract 800 mgL^{-1}
A1R0 : IAA 0.5 mgL^{-1} and yeast extract 0 mgL^{-1}
A1R1 : IAA 0.5 mgL^{-1} and yeast extract 200 mgL^{-1}
A1R2 : IAA 0.5 mgL^{-1} and yeast extract 400 mgL^{-1}
A1R3 : IAA 0.5 mgL^{-1} and yeast extract 800 mgL^{-1}
A2R0 : IAA 1.0 mgL^{-1} and yeast extract 0 mgL^{-1}
A2R1 : IAA 1.0 mgL^{-1} and yeast extract 200 mgL^{-1}
A2R2 : IAA 1.0 mgL^{-1} and yeast extract 400 mgL^{-1}
A2R3 : IAA 1.0 mgL^{-1} and yeast extract 800 mgL^{-1}
A3R0 : IAA 1.5 mgL^{-1} and yeast extract 0 mgL^{-1}
A3R1 : IAA 1.5 mgL^{-1} and yeast extract 200 mgL^{-1}
A3R2 : IAA 1.5 mgL^{-1} and yeast extract 400 mgL^{-1}
A3R3: IAA 1.5 mgL^{-1} and yeast extract 800 mgL^{-1}

Height of shoots

The results of ANOVA analysis showed that there was no interaction between IAA and yeast extract. The application of yeast extract does not affect the height of agarwood plantlets, which was contrary to Marlina et al. (2019) that yeast extracts gave significantly affected the length of shoots on mangosteen explants. It was assumed that yeast extracts contain nitrogen, vitamins, and carbon compounds that play a role in plant physiological processes. Abraham et al. (2011) showed that a yeast extract does not affect the proliferation of plant buds in vitro *C. mango*.

Table 1 showed that the application single treatment of IAA is able to affect the height of agarwood plantlets which is IAA by 0.5 and 1.0 mgL^{-1} were significantly different and had shoot high by 2.21 and 2.33, respectively. According to Bennett et al. (2016) the proper concentration of IAA could support bud growth. Moreover, the combination of yeast extract which contains amino acids (glycine, lysine, and arginine) and vitamins could encourage explant growth. However, the application of high yeast extract could inhibit plant growth (Giap et al. 2018).

In addition, this research showed that the average height on single treatment of IAA by 0, 0.5, and 1.0 mgL^{-1} have no significantly different results on the high growth of agarwood shoots. Furthermore, the application of IAA with the concentrations 0 mgL^{-1} were not significantly different from IAA by 0.5, 1.0, and 1.5 mgL^{-1} in high growth agarwood shoots. Akbar et al. (2017) argued that the length conditions of shoots of each explant are different that can be occurred because the absorption of nutrients per explant for regeneration is different, such as the growth and development of shoots.

Number of leaves

The ANOVA analysis showed that the yeast extract, as well as the interaction of IAA and yeast extracts, had no significant effect on the growth of number of leaves in agarwood plantlet. However, the treatment of single IAA has a significant effect on the growth of the number of leaves in agarwood plantlets.

Table 1. Average height of agarwood plantlet shoots at single treatment IAA concentration

IAA concentration (mgL^{-1})	Height of shoot (cm)
0	1.88 ab
0.5	2.21 a
1.0	2.33 a
1.5	1.57 b

The numbers in the column followed by the same lowercase letters did not differ noticeably according to DMRT advanced tests at a rate of 5%

Table 2. Average number of agarwood planlet leaves at a single treatment of IAA concentration

IAA concentration (mgL^{-1})	Number of leaves
0	18.25 a
0.5	20.58 a
1.0	17.75 a
1.5	10.33 b

The numbers in the column followed by the same lowercase letters did not differ noticeably according to DMRT advanced tests at a rate of 5%

Table 2 shows that the average number of agarwood plantlet leaves at a single IAA treatment with the concentration by 0 mgL^{-1} was not significantly different against IAA by 0.5 and 1.0 mgL^{-1} but it was significantly different against the application of IAA by 1.5 mgL^{-1}. Concentration of 1.5 mgL^{-1} IAA the number of leaves is significantly reduced (Figure 5). The higher the IAA concentration in the treatment medium will spur the growth of taro leaves satoimo but if the concentration of IAA has exceeded the optimum point, it will inhibit the growth of the leaves and produce fewer leaves (Louw et al.2018). Akhiriana et al. (2019) revealed that application of IAA by 0.25 ppm can produce the highest number of leaves in *Tribulus terrestris* L. Suparjo et al. (2016) declare that application of IAA more than 0.5 ppm can decrease the number of leaves, the number of segments, and the number of branches on binahong plants in vitro. Moreover, treatment with MS + 2 mgL^{-1} BA + 0.1 mgL^{-1} IAA media produces the highest average number of shoots on tomato explant and it is also the best combination for the regeneration of tomato cotyledon and hypocotyl explants (Gerszberg et al. 2016). The results showed that the difference in response Jabon of treatment tested was the highest number of buds and leaves were in the media added 0.1 mgL^{-1} IAA + 1.5 mgL^{-1} BAP (Taiyeb and Baharuddi 2017).

Number of roots

The number of roots from 64 plantlets sub-cultured is only 5 plantlets could be roots formatting (Figure 6). It is suspected because the ability of plantlets to form roots varied. Moreover, the solidity is assumed to be another factor of root formation when the media is too solid due to the excessive agar content of the MS media and yeast extract. Arab et al. (2018) revealed that potassium by regulating osmotic potential and ammonium by reducing pH in the medium could increase rooting in *Prunus domestica*. Different genotypes of agarwood plants will provide different morphological and anatomical growths, including roots (Satria et al. 2017). These results are contrary to Saikia and Karuna's research (2015) which showed that the increase in auxin concentration was positive for the number of roots, but negatively affected the length of the roots in agarwood. Lower IAA concentration (0.5 mgL^{-1}) led to a significant reduction or number of strawberry roots (4.83 roots/explanation) (Danial et al. 2016). Putri et al. (2015) argue that when yeast interacts in the media, the content of Nitrogen compounds in yeast can interfere with the growth of roots in planlets. Mastur et al. (2015) state that too high nitrogen content can cause poisoning in plants.

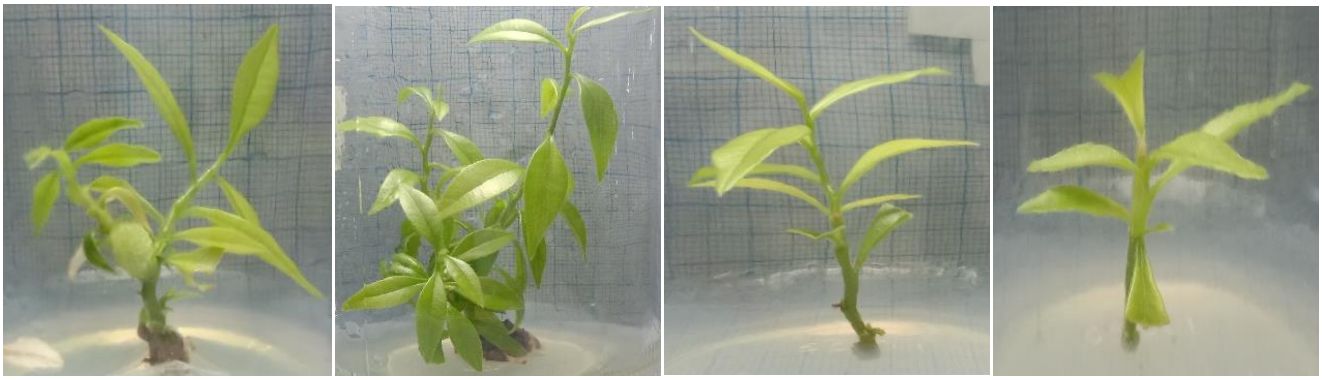

Figure 5. Agarwood plantlet leaves IAA. A. 0 mgL^{-1}. B. 0,5 mgL^{-1}. C. 1 mgL^{-1}. D. 1,5 mgL^{-1}

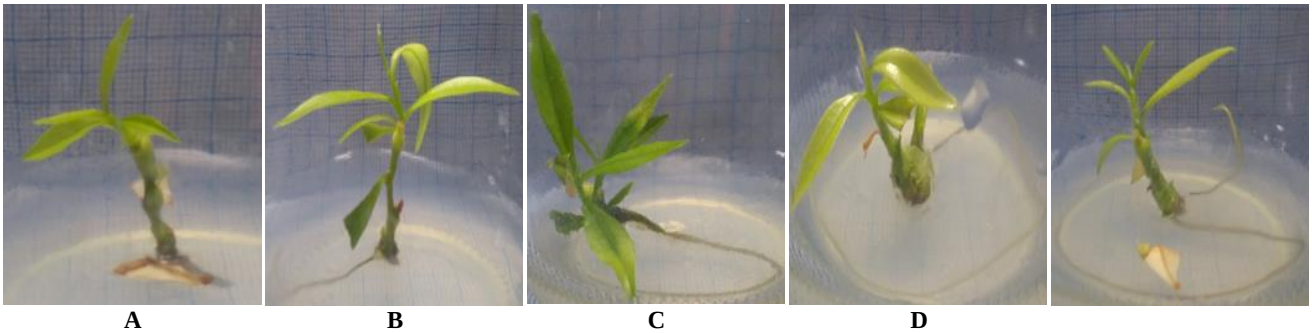

Figure 6. The appearance roots on the plantlet. A. IAA 0,5 mgL^{-1}+yeast extract 200 mgL^{-1}. B. IAA 0,5 mgL^{-1}+ yeast extract 800 mgL^{-1}. C. IAA 1 mgL^{-1}+ yeast extract 200 mgL^{-1}. D. IAA 1 mgL^{-1}+ yeast extract 400 mgL^{-1} at planlet 1. E. IAA 1 mgL^{-1}+ yeast extract 400 mgL^{-1} at planlet 2

Table 3. Combination of IAA and yeast extract treatment against roots formation

Number	Treatment combination		Number of roots
	IAA (mgL^{-1})	Yeast extract (mgL^{-1})	
1	0,5	200	1
2	0,5	800	1
3	1	200	1
4	1	400	2
5	1	400	1

Table 3 shown that 1 root were formatting on the combination treatment of IAA by 0.5 mgL^{-1}+yeast extract by 200 mgL^{-1}; IAA by 0.5 mgL^{-1}+yeast extract by 800 mgL^{-1}; and IAA 1.0 mgL^{-1}+yeast extract 200 mgL^{-1}, respectively. Moreover, 2 roots with the highest results were formatting on the combination treatment of IAA 1 mgL^{-1}+yeast extract 400 mgL^{-1} (Table 4). The results Miri's (2020) were obtained from 2 mg/l of IAA (6.6 roots per bud). Research Dasri et al. (2016) shows the highest number of orchids roots (3.20 roots/plants) was obtained when cultured on MS medium supplemented with 0.25 mgL^{-1} IAA produced by CF-B. Bogale (2018) states that low IAA concentrations can form strong roots. Miri's research (2020) showed that IAA 2 mgL^{-1} has a large number of roots with an average of 6.6 roots per bud in *Zingiber officinale*. IAA with concentrations of more than 0.5 mgL^{-1} can inhibit the growth of the roots of *Brassica napus* L. (Xie et al. 2020). Prabowo et al. (2018) state that media treatment significantly affected the number of the root that the MS medium was capable of formatting roots with the best result of 3.67 roots per piece plantlet. Rachmi et al. (2020) declare that application of MS media enriches with papaya extract was resulting number of roots by 7.3 roots per piece of plantlet. The addition of 50-100 mlL^{-1} concentration of coconut water to MS media induces the formation of number of roots and root lengths (Mardhikasari et al. 2019).

In conclusions**,** the application of combination of IAA and yeast extract have not shown an increase in the growth of agarwood planlets as in vitro. The IAA concentrations by 0,5 mgL^{-1} is able to increase the height of agarwood shoots. Furthermore, IAA concentration by 0 mgL^{-1} has the highest average number of agarwood leaves. Concentrations of yeast extract (0 mgL^{-1}; 200 mgL^{-1}; 400 mgL^{-1}; 800 mgL^{-1}) were not shown the agarwood plantlet growth. Not all treatments formatting the roots, only IAA treatment 0,5 mgL^{-1}+yeast extract 200 mgL^{-1}; IAA 0.5 mgL^{-1}+yeast extract 800 mgL^{-1}; IAA 1 mgL^{-1}+yeast extract 200 mgL^{-1}; IAA 1 mgL^{-1}+yeast extract 400 mgL^{-1}.

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