Embryogenic Callus Induction of Aquilaira Malaccensis Lam. and Aquilaria Subintegra Ding Hou

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Abstract*: *Aquilaria malaccensis Lam. and Aquilaria subintegra Ding Hou belong to the family of Thymelaeaceae which is commonly known as gaharu or agarwood. It is a commercially important tree and identified as a potential aromatic plant. The overwhelming responses in the lodging sector reduce gaharu species in the forest. Mass propagation through plant tissue culture technology will substitute this problem. The present study was conducted to investigate the embryogenic callus induction between these two species. The most optimum sterilization method for both species was sodium hypochlorite 5.0% which gave the highest percentage of aseptic culture (95%) with the absence of tissue browning. The leaves of both species were cultured on Murashige and Skoog, (1962) (MS) media supplemented with combination of various concentrations of 6-benzylaminopurine (BAP) (0.5, 1.0, 2.0 and 2.5 mg/L) and 2,4-dichlorophenoxyacetic acid (2, 4-D) (0.5, 1.0, 1.5 and 2.0 mg/L) and kept under dark condition. The explants produced embryogenic, white and compact callus at the end cut of the explants after two weeks of culture in all treatments. The highest frequency of embryogenic callus formation was observed in explants cultured on 2.0 mg/L BAP and 0.5 mg/L 2,4-D for both species. From the present study, the optimum sterilization technique and embryogenic callus induction for A. malaccensis Lam. and A. subintegra were established.

Keywords* : *Aquilaria malaccensis Lam., embryogenic callus induction, gaharu, somatic embryogenesis.

I. INTRODUCTION

Aquilaria malaccensis Lamk. and *Aquilaria subintegra* Ding Hou are the scientific name of agarwood, located under Aquilaria genus of the plant family Thymelaeaceae. Other common names of these plants are aloeswood and eaglewood. The vernacular names are gaharu and karas which are well known in Indonesia and Malaysia. Gaharu is the dark resinous heartwood which is the fragrance secreted under the bark of the plants. The gaharu formed as a response to immune reaction towards injury, trauma or infection caused by fungi and other microbes. The compositions of gaharu mainly come from two compounds which are sesquiterpenes and phenylethyl chromone derivatives. It has been reported that, a great variety of sesquiterpenes are contained in high quality agarwood [1]-[5]. However, not all Aquilaria plants could produce gaharu but only a certain species approximately 10% of wild Aquilaria spp. could produce them [6]. The resinous portion of the agarwood makes them very valuable for human being.

The gaharu is mainly used in medicinal components in oriental medicine, perfumes in the Arab world and incense for religious purpose and ceremony for many other cultures [7], [8]. Agarwood is one of the main important ingredients in the perfume industry. The strong and long lasting fragrance from the agarwood makes them more likely to be used on the human body, clothes, houses and also to honored the guests. Chinese used the agarwood to make incense, burned them for ritual and other religion purpose. According to [9], agarwood is very effective in medicinal purpose including for healing dropsy, as a carminative, a stimulant, for heart palpitations. [10] had reported that, agarwood oil is rich in sesquiterpenes compound which very effective for anti-microbial activity against most gram-positive bacteria such as *Staphylococcus epidermidis* and *Bacillus subtilis*. *Aquilaria sp*. is commonly propagated through seeds which germinate readily after maturity. However, the rate of germination sharply decreases with the increase in the period of storage or decrease in its moisture contents. Fresh seeds obtained by splitting open the capsule showed a short dormancy period ranging from 1 week to 4 weeks only. The maximum germination (90%) takes place within 20 days in case of fresh seeds and 7 days for stored seeds with very less germination percentage. Seed weight has a positive influence in germination, plant size, root length and ultimately in yearly plant establishment [11].

Overwhelming response in the lodging sector gave a very bad impact on *Aquilaria sp*. plants including *A. subintegra* and *A. malaccensis*. The source of agarwood had become scarce nowadays due to its uncontrolled collection in the forest. One of the methods to overcome these problems is by the application of plant biotechnology through in vitro techniques. In vitro propagation method is the best way to conserve and multiply endangered plant species [12]-[14]. Other than that, an urgent method is needed to make sure regeneration and conservation of this species sustainable [15].

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Recently, application of biotechnology in the plant tissue culture especially in plant in vitro regeneration, mass propagation and in vitro conservation of rare and endangered species were successfully established [16]-[17]. In vitro regeneration of techniques has been popular methods to conserve the endangered species. Callus formation is one of the important aspects in plant biotechnology and considered to be among the most important subject in micropropagation technologies [18]. Callus resulted from culturing of explants on callus induction media will produce regeneration of plant through the process of organogenesis or somatic embryogenesis [5]. Callus is mass of unorganized parenchyma cells derived from plant tissue and in plant biology it refers to cells that cover a plant wound. To initiate and maintain the callus, mainly 2, 4-dichlorophenoxy acetic acid (2,4-D) is used alone or in combination with cytokinin [19]. Other than that, genetic factors are one of the major factors that influence the in vitro response of plant tissue culture. Genotype and source of explants would influence the production of embryogenic calli and also the regenerated plantlets [20]. Many reports on successful embryogenic callus induction in many plant species using 2,4-D and BAP hormone have been reported [21]-[24].

II. PROCEDURE

A. Plant Materials

Surface sterilization is the primary establishment of aseptic *in vitro* culture taken from the field or environment. The fresh leaves of *A. malaccensis* and *A. subintegra* (3 months old) were collected from the Gaharu plantation in Selangor and Melaka.

B. Sterilization Procedure

The leaves collected from the field were washed under running tap water for 30 minutes and followed by soaking in detergent for 15 minutes. All the leaves were rinsed with sterile distilled water for 3 times and transferred into the laminar air flow for further sterilization techniques. Then, all explants were rinsed with 70% ethanol for 20 minutes. After that, 4 methods of sterilization were tested on the leaf explants where 0.1% mercury (II) chloride, $HgCl_2$, for 5 minutes and three concentrations of sodium hypochlorite, NaClO, (2.5%, 5.0% and 10.0%) with a few drops of tween 20 for 20 minutes. A group of control explants were sterilized using only 70% ethanol.

C. Media Preparation

An amount of 4.4 g/L of MS media [25] with 30.0 g/L sucrose and 2.0 g/L gelrite were used as the basal medium. Combination of 2,4-D (0.5, 1.0, 1.5 and 2.0 mg/L) and (0.5, 1.0, 2.0, 2.5 mg/L) BAP were added into the MS basal medium. The pH was adjusted to 5.7-5.8 using NaOH and HCl and followed by autoclaved at 121°C and 1.05 kg/cm3 for 20 minutes.

D. Embryogenic Callus Induction

The sterilized leaves were cut into pieces (approximately $1cm^2$) and then inoculated onto the prepared MS media supplemented with various combination of 2,4-D and BAP. All cultures were kept in the dark with environment temperature of 25 ± 1°C. MS media without plant growth regulator was served as control. Subcultures were done every 4 weeks interval and observation was done weekly.

E. Analysis of Result

The assessment on embryogenic callus induction of *A. malaccensis* and *A. subintegra* was done through qualitative and quantitative analysis. Qualitative analysis was analyzed by observing the morphological characteristics of the cultures. Other than that, quantitative analysis was conducted by calculating the percentage of callusing (%), percentage of browning (%), callus induction rate (%), weight (g) and degree of the callus formed. The data were collected every 4 weeks of inoculation.

III. RESULTS

A. Optimization of Sterilization Method

From the observation, all explants for both species in the control group were contaminated after a few days, giving a contamination rate for control group is 100% by day 15 (Table-I). After 15 days, contamination rate for 0.1% $HgCl_2$ was 1% for both *A. malaccensis* and *A. subintegra*. However, most of the explants lost their viability, degenerated and showed browning of the explants with rate of 90% browning. Using 0.1% of $HgCl_2$ as sterilization agent gave higher percentage of aseptic culture, hence higher percentage of sterility, toxic and browning. Higher concentration of NaClO as the sterilizing agent would give higher percentage of aseptic culture while lower concentration would give opposite result. Higher concentration of NaClO showed higher percentage of aseptic culture which is more than 90% but also caused severe damage to the explants for both species. Besides, lower concentration of NaClO gave less percentage of contamination 5% without any browning effect on the explants culture for both species.

Table-I The effect of four sterilization treatments on the percentage of aseptic culture and browning of *A. malaccensis* and *A subintegra* after 6 weeks on MS media.

Sterilization treatment	*A. malaccensis*		*A. subintegra*	
	Aseptic culture (%)	Browning (%)	Aseptic culture (%)	Browning (%)
0.1% $HgCl_2$	100	90	100	90
2.5% NaClO	34	-	40	-
5.0% NaClO	95	-	95	-
10.0% NaClO	95	50	95	30
MS0 (Control)	-	-	-	-

B. Establishment of Embryogenic Callus Induction

After 7 days of culture, the explants were started to swell and produced white, translucent and friable callus after 12 to 14 days of culture for both species and produced both yellow and white calli. The formation of white callus might be due to the absent of light.

The result showed that combination of both BAP and 2,4-D hormones generally favors callus initiation and formation. Callus induction rate (%) for both species in response to different combinations of auxin and cytokinin in MS media is presented in Table-II and III.

Calli were developed in all hormonal combinations for both species, with various callus induction frequencies which were ranging from 86% to 100%. Average fresh weights of callus were determined after 45 days in culture.

Table-II Effect of plant growth regulators on callus induction frequencies and weight of *A malaccensis* after 45 days.

Treatments	PGR combination (mg/L)		Callus induction rate (%)	Average fresh weight (g)	Callus characteristics	Degree of callus formation
	BAP	2,4-D				
1	0.5	2.0	100.0	0.7212	White and compact	+++
2	1.0	2.0	100.0	0.0413	White, friable and smooth	+
3	2.0	2.0	100.0	1.4820	Yellowish white and compact	++++
4	2.5	2.0	100.0	0.4542	White and compact	++
5	0.5	1.5	100.0	0.6496	White and compact	+++
6	1.0	1.5	100.0	0.7308	White and compact	+++
7	2.0	1.5	100.0	0.6789	White and compact	+++
8	2.5	1.5	100.0	0.5441	Yellowish white and friable callus	+++
9	0.5	1.0	100.0	0.5671	White and compact	+++
10	1.0	1.0	100.0	0.4724	White and compact	+++
11	2.0	1.0	100.0	0.6597	Yellowish white and friable callus	+++
12	2.5	1.0	100.0	1.4595	White and friable callus	++++
13	0.5	0.5	86.7	0.2785	White and compact	++
14	1.0	0.5	95.0	0.3143	White and compact	++
15	2.0	0.5	100.0	1.7922	Yellowish white and friable callus	++++
16	2.5	0.5	100.0	1.5567	White and friable callus	++++
Control	0.0	0.0	-	-	-	-

-: No calli; +: Very few calli; ++: Minor calli; +++: Moderate calli; ++++: Profuse calli

Table-III The effect of plant growth regulators on callus induction frequencies and weight of *A. subintegra* after 45 days.

Treatment	PGR combination (mg/L)		Callus induction rate (%)	Average fresh weight (g)	Callus characteristics	Degree of callus formation
	BAP	2,4-D				
1	0.5	2.0	100	0.6615	White and friable callus	+++
2	1.0	2.0	100	0.3111	White and friable callus	++
3	2.0	2.0	100	0.0577	White, friable and smooth callus	+
4	2.5	2.0	100	0.4463	White and friable callus	++
5	0.5	1.5	100	0.6013	White and compact	+++
6	1.0	1.5	100	0.6597	White and compact	+++
7	2.0	1.5	100	0.6772	White and compact	+++
8	2.5	1.5	100	0.7401	White and compact	+++
9	0.5	1.0	100	0.4775	White and friable callus	+++
10	1.0	1.0	100	0.5182	White and friable callus	+++
11	2.0	1.0	100	0.4953	White and friable callus	+++
12	2.5	1.0	100	0.5073	White and compact	+++
13	0.5	0.5	90	0.4464	White and compact	+++
14	1.0	0.5	95	0.3979	White and compact	+++
15	2.0	0.5	100	1.5454	White and compact	++++
16	2.5	0.5	100	1.4033	White and compact	++++
Control	0.0	0.0	-	-	-	-

-: No calli; +: Very few calli; ++: Minor calli; +++: Moderate calli; ++++: Profuse calli

The highest callus weight was recorded with 1.7922 g for *A. malaccensis* and 1.5454 g for *A. subintegra*. The lowest weight for *A. malaccensis* was obtained with 0.6131 g while 0.0577 g was recorded for *A. subintegra*. Most of the calli produced were white and compact. Some treatments produced white/yellowish white and compact calli. In contrast, six treatments from *A. malaccensis* and seven treatments from *A. subintegra* produced friable calli. The combinations of high concentrations of both 2,4-D and BAP in *A. malaccensis* produced compact calli. In *A. malaccensis*, high 2,4-D concentration (1.5 and 2.0 mg/L) with various BAP concentration mostly promoted the formation of embyogenic, white and compact calli except treatments 3 and 8 which produced friable calli. On the contrary, most of the calli produced in *A. subintegra* were friable calli in 2.0 mg/L 2,4-D with various BAP concentrations. In *A. malaccensis*, combination of high BAP concentration (2.0 and 2.5 mg/L) with various 2,4-D concentrations mostly promoted compact friable calli except for treatment 3, 4 and 7. Meanwhile, *A. subintegra* produced mostly compact calli except for treatment 3, 4 and 11.

Lowest concentration of 2,4-D and BAP resulted white and compact calli for both species. Lower concentration of 2,4-D at 0.5 mg/L with higher concentrations of BAP (2.0, 1.5 & 1.0 mg/L) in *A subintegra* promoted the formation of white and compact calli (Fig 1). In contrast with the findings in *A. subintegra*, calli produced from such combinations of treatments in *A. malaccensis* were white and friable (Fig. 2).

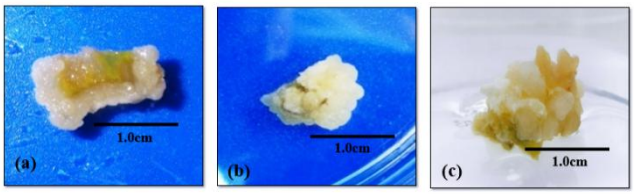

Fig.1: Leaf-derived calli of *A. malaccensis* maintained in MS medium supplemented with 2.0mg/L BAP + 0.5mg/L 2,4-D after (a) 14 days; (b) 30 days and (c) 45 days of culture.

Fig. 2: Leaf-derived calli of *Aquilaria subintegra* maintained in MS medium supplemented with BAP 2.0mg/L + 2,4-D 0.5mg/L after (a) 14 days; (b) 30 days and (c) 45 days of culture.

The lowest concentrations of 2,4-D and BAP at 0.5 mg/L each resulted in the formation of white and compact calli in both species.

Meanwhile, highest concentrations of BAP (2.5 mg/L) regardless of 2,4-D concentration (except 2.0 mg/L) mainly produced white and friable calli in *A. malaccensis*. This was seen to be on the contrary to the findings found in *A. subintegra* whereby such combinations resulted in the productions of white and compact calli. From the present experiments, the best hormonal combination was found in the MS media supplemented with BAP 2.0mg/L + 2,4-D 0.5mg/L for both species due to the highest fresh weight with desirable characteristics of callus.

IV. DISCUSSIONS

A. Optimization of Sterilization Method

Aquilaria sp. was very important plant due to its resin and aromatic compound that have high value in the market. However, limited number population and conventional breeding methods that take times necessitate the propagation of the species to be done through in vitro culture. Contamination is one of the biggest constrain happen in plant tissue culture protocols. Contaminations mainly by fungus and bacteria affect the percentage of valuable aseptic culture. The present study was carried out to find optimum sterilization protocol for *A. malaccensis* and *A. subintegra*. Using 0.1% of $HgCl_2$ as sterilization agent gave higher percentage of aseptic culture, hence higher percentage of sterility, toxic and browning. This might be because of HgCl2 is a strong a chemical although used in very low concentration (0.1%) and prolonging of soaking time of explants in the solution which might give harm and toxicity to the explants.

It was discovered that the treatment which used 5.0% NaClO for 20 minutes was the best methods to optimize sterilization in *A. subintegra* leaves culture with less contamination rate and zero browning. Moreover, careful selection of explants from the healthy and quality parent plants combined with effective surface sterilization techniques should be the best approach to avoid contamination in the culture [26].

Selection of explants from the field grown plants would not be an easy job due to presence of contaminant especially on the surface and inside the plant tissue. To avoid contamination, surface sterilized must be conducted; however, systemic contaminants could not be avoided and could not be removed [26]. This can be explained by the presence of contamination of the explants in the culture even though serials sterilization and stronger chemicals has been used to sterilize the explants. A common sterilizing agent used in many plant tissue culture techniques is NaClO. According to [7], 1.4% of NaClO for one minute was the effective concentration for leave sterilization of *A. crasna* and *A. sinensis*. Meanwhile, 50% bleach was the best sterilizing agent for shoot tips and nodal explants from young seedling of *A. hirta* [27]. Previous studies have found that, application of NaClO as the sterilizing agent is effective for *A. malaccensis* [28]-[30].

Besides using NaClO, $HgCl_2$ at a very low concentration has also been effectively used as sterilant in sterilization of in vitro propagation of *A. agallocha* [31] and *A. crassna* [32]. However, in this study, the application of mercury (II) chloride as sterilizing agent was unsuitable and caused necrosis to the explants tissues. This is most probably due to the nature of the tissue of the stock plants which were derived from young leaves which could not withstand the strong chemical. Other than that, [33] claimed that, exposure of mercuric (II) chloride to explants will lead to reduction of survival rate of the explants. In addition to that, [34] stated that prolong mercuric chloride treatment will cause blackening and subsequent death of explant, thus the duration of treatment is critical. Nevertheless, there are still many reports on sterilization methods using mercury (II) chloride as their sterilizing agent [35]-[37].

Effectiveness of sterilization method in plant tissue culture is not only depends on the type and concentration of sterilizing agent used but also duration of time applied. In addition, plant species used also being part of significant factors which determine the effectiveness of sterilization method. Mainly, there were two types of sterilizing agent used which were ethanol and sodium hypochlorite. Severe contamination would need stronger chemicals such as $HgCl_2$ to reduce and avoid contamination. Ethanol is a strong chemical and normally used after dissolved in distilled water. According to [34], alcohols are rapidly bactericidal, tuberculocidal, fungicidal, and virucidal, unfortunately they cannot destroy bacterial spores. The optimum concentration for alcohol to kills microbes were between 60%-90%, however their toxicity falls sharply when diluted lower than 50% in distilled water [34], [38]. Due to high phytotoxicity factor, this chemical can only be used in short time. As well as sodium hypochlorite, this chemical is proven to be very successful in killing microbes even though in very low concentration. [39] & [40] had reported that, when hypochlorite salt (NaOCl) diluted in water, they will produce HClO compound that exploit the microbe activity and caused lethal DNA damage and caused the death of the microbes.

B. Establishment of Callus Induction

The cultures were initiated from the leaves of *A. malaccensis* and *A subintegra* in MS media supplemented with various concentration of BAP and 2,4-D hormones (Fig. 1 and Fig. 2). [41] reported that leaves were the best explants for callus induction and growth characteristics of callus for *Aquilaria sp*. therefore, leaves were used as the explants for callus induction in this experiment.

The presence of light promotes the synthesis of chlorophyll that will cause the green colour of the callus [42], [43]. However, in this situation, the light was absent and synthesis of chlorophyll could not be produced therefore, none of the calli produced were green. The callus mainly formed at the cut area and edge of the explants. There was no callus formation observed on control media.

Cytokinins were important for callus differentiation and the ratio of the auxin to cytokinin determining the type of culture established or regenerated. Low auxin to cytokinin ratio results in shoot formation, intermediate ratio results in callus formation and high ratio induces root formation [44]. The best hormonal combination was identified from the treatment which produced the highest fresh weight with desirable characteristics of callus.

Higher fresh weight indicates the higher amount of biomass cell in the callus. There were so many similar results have been reported [29], [44], [45]. Callus cultures were extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced. Callus can also be used to initiate cell suspension cultures which are useful in plant transformation studies [46].

Generally, there were no much differences in callus formation and initiation between these two species. The characteristics of the callus formed in these two species were almost similar. This is probably because they derive from the same family and having similar habitat, hence producing similar responses toward treatments given. Explants responses towards different treatments were found to be similar as well. Upon observation by scanning electron microscopy (SEM), rapidly dividing undifferentiated cells could be seen clearly in both *A. malaccensis* and *A. subintegra* (Fig. 3).

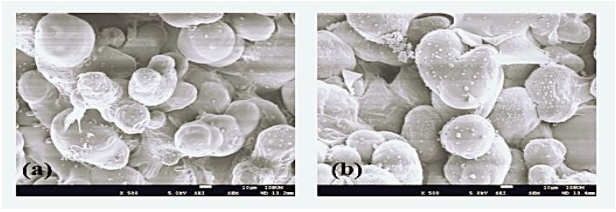

Fig. 3: Image of actively dividing cells of embryogenic callus of (a) A. malaccensis and (b) A. subintegra viewed by Scanning Electron Microscope under 500X magnifications.

V. CONCLUSION

In this study, the sterilization technique for *A. malaccensis* and *A. subintegra* was optimized and a reproducible, standard and efficient tissue culture protocol for embryogenic callus initiation using leaf explants of *A. malaccensis* and *A. subintegra* has been defined. Further studies on hormones and other conditions should be done to increase the quality and quantity of embryogenic callus and initiation of somatic embryoids that can be expanded to protoplast culture, cell suspension culture and genetic transformation.

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