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Research Article
Establishment of Callus and Cell Suspension of *Aquilaria rugosa* L.C. Kiet and Keβler in Vietnam

[1]Nguyen Thi Tho, [1]Nguyen The Nha, [1]Nguyen Thanh Tuan, [1]Hoang Thi Hang, [1]Hoang Van Sam, [1]Ha Van Huan, [1]Phung Thi Tuyen, [2]Pham Bich Ngoc, [3]Rusteberg Jan-Philip, [3]Olarte Alexandra and [3]Cerboncini Claudio

[1]Vietnam National University of Forestry, Ha Noi, Vietnam
[2]Institute of Biotechnology, Vietnam Academy of Science and Technology, Vietnam
[3]Institute for Bio- and Geoscience, IBG-2: Plant Sciences, Forschungszentrum Jülich GmbH, Germany

Abstract

Background and Objective: *Aquilaria rugosa* L.C. Kiet and Keβler (Thymelaeaceae family) species is considered to be endangered in its original mountainous forest habitats. This study was conducted to establish a protocol of callus and cell suspension of *A. rugosa*. **Materials and Methods:** Three healthy plant parts of *A. rugosa* including leaves, stems and roots of *in vitro* 2 months old seedlings were used as explants for callus induction in Murashige and Skoog (MS) medium with 2.0% sucrose and 0.8% phytoagar at pH = 5.7. The growth regulators were tested at different concentrations (0.0-4.0 mg L^{-1}) for Indole Butyric Acid (IBA), Indole Acetic Acid (IAA), a-naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP) and 0.5-1.0 mg L^{-1} for BAP to find out which types of growth regulators were the most efficient for inducing callus of *A. rugosa*. **Results:** The research indicated that leaves, stems and roots of *in vitro* 2 months old seedlings were suitable for callus induction. The combination between auxin and cytokine was better than using single auxin in callus formation. Murashige and Skoog's (MS) medium supplemented with 2 mg L^{-1} IBA, 1 mg L^{-1} BAP was the most efficient for inducing callus of *A. rugosa* (108.53±4.47 mg FW/leaf explant). The maximum growth rate of callus was observed in the 4th week of culture on the same maintenance media. Effective establishment of *A. rugosa* cell suspension was recorded with 2 g of initial callus fresh weight and in sucrose concentrations 40 g L^{-1}. **Conclusion:** The research also suggested that medium supplemented with 2 mg L^{-1} IBA,1 mg L^{-1} BAP was the most suitable for inducing callus and initial inoculum sizes and sucrose concentration had effects on the growth of cells *A. rugosa*.

Key words: *Aquilaria rugosa*, callus induction, cell suspension, BAP, IBA

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Corresponding Author: Nguyen Thi Tho, College of Forestry Biotechnology, Vietnam National University of Forestry, Xuan Mai Town, Chuong My District, Hanoi 10000, Vietnam Tel: +84 973954911

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aquilaria rugosa L.C. Kiet and Keβler was first discovered in 2005 in Sa Thay district, in the province Kon Tum of Vietnam[1]. It was previously assumed to be an endemic *Aquilaria* species of the Vietnamese central highlands of Kon Tum province. However, it also occurs in northern highlands of Thailand and its distribution range has been suggested to spread into Lao[2]. This species is considered to be endangered in its original mountainous forest habitats[1]. However, in Kon Tum province it is successfully domesticated and sporadically planted in private home gardens and local community agro forestry's where mature trees employed to produce resinous fragrant wood by artificial inducements[1]. *A. rugosa* is officially called mountain agarwood and is for its natural habitat in mountainous regions above 500 m.a.s.l considered to be superior to the common lowland *Aquilaria crassna,* mostly due to a better overall grow performance.

In general, agarwood from that *Aquilaria* sp. was well-known with high value of non-timber forest product. Agarwood has been traditional used in perfumery, decoration, meditation and other religious purposes in many countries of the world[3]. Moreover, it also was a natural source for medicines and health products[4,5]. It is demonstrated that agarwood is formed in the heart wood in response to injury and fungal infection[6,7]. Due to the high value of agarwood, *Aquilaria* species have been overlogging in Vietnam for a long time. Thus, the populations of most *Aquilaria* species have been significantly decreased in the wild.

High plants are considered as rich sources of secondary metabolites with high medicinal values[9]. The secondary metabolites in plant cells or organ cultures established from biotechnology are potential extraction material of whole plant[8,9]. There are two types of tissue culture technique including unorganized (callus and suspension cultures) and organized (root cultures, shoot tip culture and embryo cultures etc.)[9]. Callus could be derived from different types of explants and culture conditions. The same explant may form calli with variation of color (light or dark), dry or wet, compact or friable. Subculture on fresh medium and rapid cell division can lead to the formation of more friable callus is highly desirable for establishing cell suspension culture[10]. Callus cultures have many applications: provide the material for establishment single cell or cell suspension, study various morphogenetic and physiological, generate useful somaclonal variations and produce secondary metabolites[10]. Calli and cell suspension culture could be used to produce sesquiterpenes in *Aquilaria*[3,11]. Sesquiterpenes is one of the principal compounds determine fragrance property and quality of agarwood[8,11].

In order to remove the pressure from wild populations and support for conservation and development of *Aquilaria* species, the cultivation and research on the establishment of tissue culture techniques has been paid more attention in Vietnam. This study aimed to establish an effective protocol for inducing callus and cell biomass multiplication of *A. rugosa* for studies of secondary metabolite production.

MATERIALS AND METHODS

Plant materials: Seeds of *A. rugosa* (were collected from Sa Thay district, Kon Tum province), Vietnam in June 2018 were germinated in Murashige and Skoog (MS) medium[12] without hormone supplemented with 2.0% sucrose and 0.8% phytoagar. Healthy leaves, stems and roots from *in vitro* 2 months old seedlings were used as explants for callus induction.

Culture media: The MS medium was used at full strength as the basal medium. MS medium and all phytohormones are purchased from Duchefa Biochemie. The pH of medium was adjusted to 5.7±0.1 with NaOH 1N or 1M HCl and then the medium was autoclaved at 121°C for 20 min. The cultures were then incubated at 26±1°C in total darkness.

Callus induction and multiplication

Effect of plant growth regulators on callus induction from leaves: The phytohormones NAA, IBA and IAA were used individually at different concentrations (from 0.0-4.0 mg L^{-1})or in combination with 0.5-1.0 mg L^{-1} of BAP to estimate the formation and growth of *A. rugosa* callus from *in vitro* leaves. Leaves were cut in 1 cm^2 pieces and placed on MS medium containing 20.0 g L^{-1} sucrose, 8.0 g L^{-1} phytoagar and different phytohormones. The capacity of callus induction was considered via color, morphology, fresh weight after 4 weeks of cultures.

Effect of different types of explants on callus formation: In order to find the effect of type of explants on callus induction of *A. rugosa in vitro* leaves, stems and roots of *A. rugosa* were cut into about 1 cm^2 pieces (leaves) or 1 cm length segments (for stems and roots) and cultured onto solid MS medium supplemented with 2 mg L^{-1} IBA and 1 mg L^{-1} BAP (the best combination of previously tested phytohormones). After 4 weeks of culture, the induction of calli in per flash was recorded[13].

Callus multiplication and maintenance on solid media: Callus derived from leaves were maintained on MS medium

supplemented with 2 mg L^{-1} IBA and 1 mg L^{-1} BAP in the dark condition to form friable callus for cell suspension culture. Fresh weigh of callus were harvested once every 7 days to find growth curve of cell callus.

Establishment of cell suspension cultures

Effect of initial callus weight on growth of cell suspension: Cell suspension cultures were initiated by transfer 2-6 g fresh weight friable calli derived from leaves into 250 mL flasks containing 100 mL MS liquid medium supplemented with 2 mg L^{-1} IBA, 1 mg L^{-1} BAP, 20.0 g L^{-1} sucrose. Cell suspension cultures were placed on a rotary shaker with a speed of 100 rpm min^{-1} at 26±1°C in the dark. The cells were harvested from suspension cultures by filtration using a Whatman filter paper (grade 1), weigh to obtain Fresh Weight (FW) after every 4 days of inoculation. Cell growth was measured on Growth Index (GI) by the formula[5,13-15]:

$$GI = \frac{W_f - W_o}{W_o}$$

where, W_f and W_0 represent the final and initial fresh masses, respectively.

Effect of sucrose concentration on growth of cell suspension: To estimated effect of sucrose concentration on *growth of A. rugosa cell suspension*, 2 g of callus cell were cultured in 100 mL MS medium added to 2 mg L^{-1} IBA, 1 mg L^{-1} BAP and different concentration of sucrose (from 10-50 g L^{-1}), shaking speed 100 rpm min^{-1} at 26±1°C in the dark. The cells were recorded on day 20 to have fresh weight before being dried at 50°C to obtain Dry Weight (DW)[5].

Statistical analysis: All the experiments above were independently repeated three times under the same conditions. Data were analyzed using SPSS Statistic version 20. Analysis of variance (ANOVA) followed by Duncan's multiple range test for mean comparison at (p = 0.05) was used to test statistical significance.

RESULTS

Effects of phytohormone treatment on callus induction: In this experiment, the effect of different concentrations of single IAA, IBA and NAA or combination with BAP on callus induction was investigated with the leaf explants. The results showed that the leaf explants in control medium (no auxin-Fig.1a) did not form callus while leaf explants in induction medium formed callus (Fig. 1b-f). Although there was not a significant difference in callus FWs between of the different concentrations of single IAA, IBA and NAA, the callus colors were different. For IBA and NAA treatments at concentrations between 2-3 mg L^{-1} showed higher biomass and viable callus induction represented by whitish colored calli. Whereas IAA treatments displayed a lower induction rate and showed a non-viable callus induction since the calli became brown during the weeks of sub cultivation. The highest biomass of callus (FW = 56.49 mg) (Table 1) was obtained from leaf explants grown in MS medium added 2 mg L^{-1} IBA but callus was still compact (Fig. 1d). A significant increase of whitish, white, cream and friable callus was found when combining BAP between 0.5-1.0 mg L^{-1} with 2 or 3 mg L^{-1} auxins (Table 2, Fig. 2a-d). A final assessment of the callus induction using the quality parameters (Table 2) revealed a significant increase in biomass accumulation (105.83 mg) using IBA and BAP in the ratio of 2:1 (w/v).

Effect of different types of explants on callus formation: The different types of explants were cultured on MS medium supplemented with the optimal phytohormone concentration

Table 1: Effects of single auxin hormone on callus induction of *A. rugosa* from leaf explants

Auxin	Concentration (mg L^{-1})	Callus FW (mg) (Mean±SE)	Degree of callus formation	Color and morphology of callus
No auxin	0	NC	NC	NC
IAA	1	32.35 ± 2.64^{f}	+	Brownish compact
	2	43.25 ± 2.56^{cd}	++	Brownish compact
	3	53.27 ± 2.78^{ab}	++	Brownish compact
	4	45.99 ± 1.64^{bc}	++	Brownish compact
IBA	1	35.99 ± 1.67^{def}	+	Yellowish compact
	2	56.49 ± 0.99^{a}	++	Whitish compact
	3	54.38 ± 0.51^{ab}	++	Whitish compact
	4	45.83 ± 1.39^{bc}	++	Whitish compact
NAA	1	34.62 ± 2.17^{ef}	+	Brownish, compact
	2	53.98 ± 2.21^{ab}	++	Whitish compact
	3	52.01 ± 1.07^{ab}	++	Whitish compact
	4	42.63 ± 1.57^{cde}	++	Brownish, compact

NC: No callus formed, +: Very few, ++: Moderate, +++: Profuse, FW: Fresh weight, values are Means±Standard errors (SE) of three independent experiments consisting of a total of 30 explants. Means followed by the same letters are not significantly different from each other at (p = 0.05) determined by Duncan's multiple range test

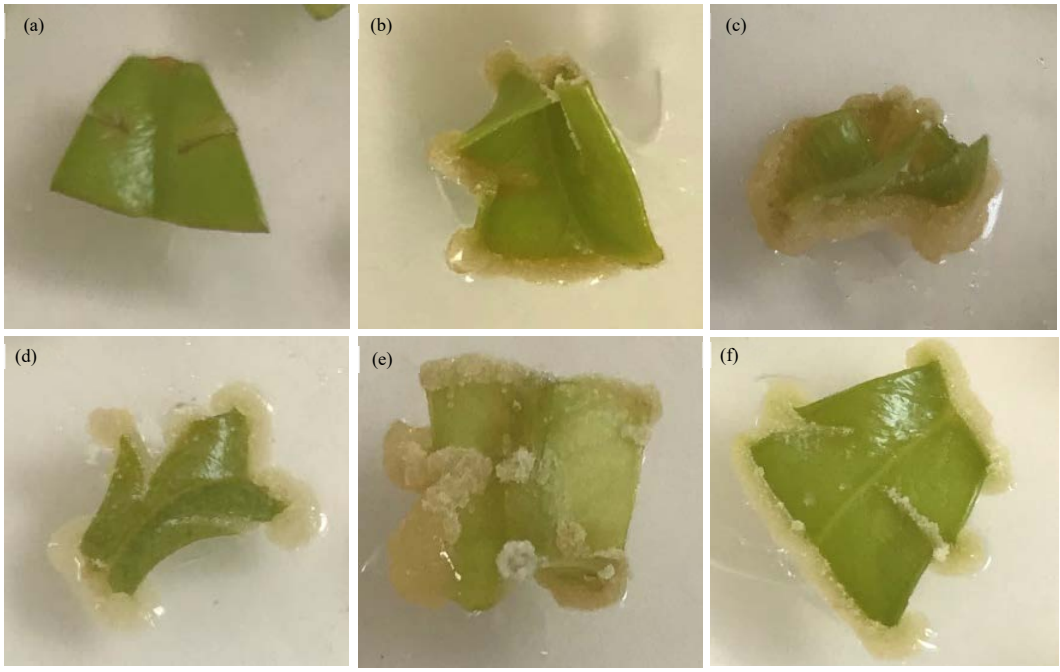

Fig. 1(a-f): Callus induction from leaf explants of *A. Rugosa* after 4 weeks in MS medium supplemented with different concentration of single auxin
(a) Control , (b) 1 mg L^{-1} IAA, (c) 3 mg L^{-1} IAA, (d) 2 mg L^{-1} IBA, (e)1 mg L^{-1} NAA, (f) 2 mg L^{-1} NAA

Table 2: Effects of combination between auxins (IAA, IBA, NAA) and cytokine (BAP) on callus induction of *A. rugosa* from leaf explants

Phytohormones (mg L^{-1})				
IAA	BAP	Callus FW (mg) (Mean±SE)	Degree of callus formation	Color and morphology of callus
0	0	NC	NC	NC
2	0.5	57.80±2.71^{de}	++	Whitish compact
2	1	67.87±8.38^{bcde}	++	Whitish friable
3	0.5	73.77±3.17^{bcde}	+++	Whitish friable
3	1	81.47±7.39^{b}	+++	Whitish friable
IBA	BAP			
2	0.5	76.90±7.13^{bcd}	++	Whitish friable
2	1	105.83±9.41^{a}	+++	Cream friable
3	0.5	66.93±8.18^{bcde}	++	Whitish friable
3	1	78.53±3.27^{bc}	++	Cream friable
NAA	BAP			
2	0.5	57.03±0.09^{e}	++	Whitish friable
2	1	86.30±4.98^{b}	+++	Cream friable
3	0.5	71.03±4.79^{bcde}	+++	White friable
3	1	59.90±1.15^{cde}	++	Whitish friable

NC: No callus formed, +: Very few, ++: Moderate, +++: Profuse, FW: Fresh weight, values are Means±Standard Errors (SE) of three independent experiments consisting of a total of 30 explants. Means followed by the same letters are not significantly different from each other at(p = 0.05) determined by Duncan's multiple range test

found previously for 4 weeks of cultivation. Table 3 shows that there are statistically three different groups of callus rate formation depending on the type explant. Induction rate with 88.89% was found in leaf explants which also reveal the highest average biomass accumulation of 108.53 mg/explant. The capability of callus formation in stem and root explants was not as high as in leaf explants. However, calli from stem explants (Fig. 3a) were more compact than from leaf (Fig. 3b) and root (Fig. 3c) tissue. These results indicated as well that leaves, stems and roots of *in vitro* seedlings of *A. rugosa* are suitable for callus induction.

Callus multiplication and maintenance on solid media: The callus multiplication and maintenance were preceded on MS

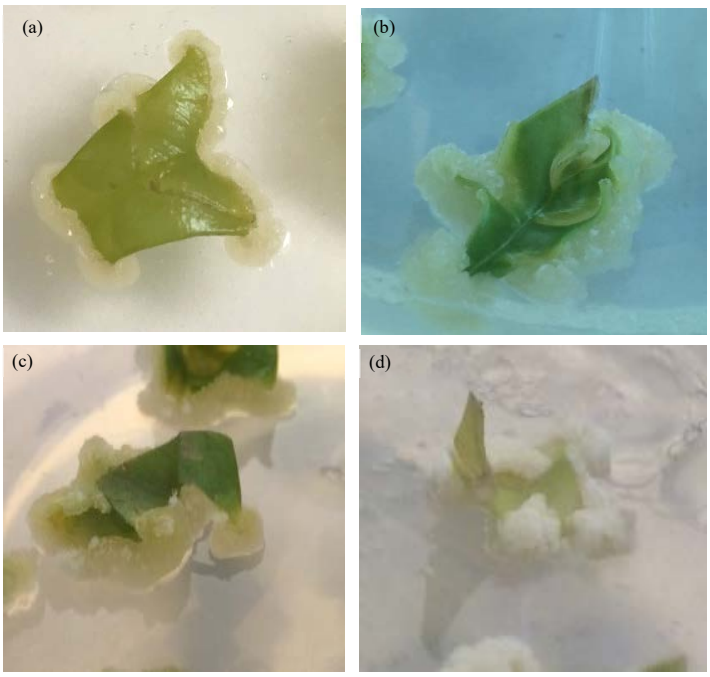

Fig. 2(a-d): Callus induction from leaf explants of *A. rugosa* after 4 weeks in MS medium supplemented with single auxin and BAP
(a) 3 IAA+1 mg L^{-1} BAP, (b) 2 IBA+1 mg L^{-1} BAP, (c) 2 NAA+1 mg L^{-1} BAP and (d) 3 NAA+0.5 mg L^{-1} BAP

Fig. 3(a-c):Callus induction from stem
(a) Leaf, (b) Root and (c) Explants of *A. rugosa* after 4 weeks in MS medium supplemented with 2 mg L^{-1} IBA and 1 mg L^{-1}BAP

Table 3: Effects of several of explants types on *A. rugosa* callus formation

Types of explants	Induction rate (%)	Callus FW (mg) (Mean±SE)	Color and morphology of callus
Leaf	88.89	108.53±4.47[a]	Cream, friable
Stem	80.00	95.45±1.05[b]	Yellowish, compact
Root	76.67	81.92±1.38[c]	Whitish, friable

Values are Means±Standard Errors (SE) of three independent experiments consisting of 30 explants. Means followed by the same letters are not significantly different at p = 0.05 using Ducan's multiple range test

medium with the optimal concentration, which yielded the highest amount of biomass (10.10 g/culture FW) after 35 days of culturing (Fig. 4). After a lag phase of 7 days the callus growth reached a log phase up to day 28, showing six times more of fresh weight than initial (1.5 g/calli FW). After peak biomass accumulation the calli moved into the stationary phase from day 28 to day 35. A decrease of callus biomass was observed on day 42 (8.71 g/calli FW) when callus clumps began to turn brown.

Effect of initial callus weight on growth of cell suspension: In order to establish an appropriate and fast-growing cell

Table 4: Effects of various sucrose concentrations on growth of *A. rugosa* cell suspension

Sucrose (g L^{-1})	FW (g)	DW (g)	GI
10	5.37±0.2[d]	0.32±0.02[d]	1.68±0.10[d]
20	6.99±0.08[b]	0.40±0.009[b]	2.49±0.04[b]
30	7.32±0.22[b]	0.41±0.009[b]	2.66±0.11[b]
40	8.52±0.22[a]	0.46±0.01[a]	3.26±0.11[a]
50	6.13±0.12[c]	0.36±0.007[c]	2.07±0.06[c]

Values are Means±Standard Errors (SE) of three independent experiments, means followed by the same letters are not significantly different from each other at p = 0.05 determined by Duncan's multiple range test, FW: Fresh weight, DW: Dry weight, GI: Growth index

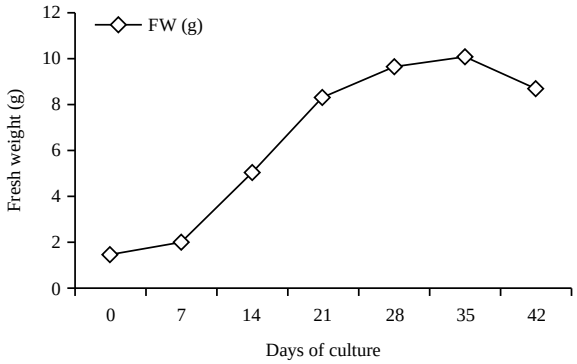

Fig. 4: Growth curve measurement of *A. rugosa* callus derived from leaf explants on MS medium supplemented with 2 mg L^{-1} IBA+1 mg L^{-1} BAP
Growth was determined every 4 days after culture

suspension, determination of optimal inoculum size is necessary. Therefore, after callus induction on solid medium and incremental FW of friable callus derived from leaf were transferred into liquid medium. There was a slight decrease of cell growth from the initial day to the end of day 4 before the exponential phase started. Suspension cultures that started with 2, 3 and 4 g of FW reach a peak of cell biomass accumulation on day 20 displaying 6.98, 8.17 and 9.69 g/culture, respectively (Fig. 5a-c).In the case of the suspension established with 5 and 6 g of initial callus, the best growth rates of cell were achieved on day 16 with 11.59 and 12.73 g (FW), respectively (Fig. 5d, e). In addition, color of cell suspension cultures using higher starting FW (5 and 6 g) became brown on day 20 (Fig. 6a) while cell suspensions with 2, 3 and 4 g never showed this appearance during the experiments (Fig. 6b). The results showed that the highest number of growth index (2.5) was found after 20 days for the cell suspension cultures initiated with 2 g of friable callus (Fig. 6c).

Effect of sucrose concentration on growth of cell suspension: The results in Table 4 showed that different sucrose concentration affected significantly on the cell suspension *A. rugosa*. In the low concentration (10 g L^{-1}sucrose), the cell grew slowly (only 5.37 g FW with GI 1.68). There was an enhancement of cell growth when increasing sucrose concentration. The highest growth of cell was found in medium supplemented with 40 g L^{-1} with 8.52 g fresh weight (Fig. 6c) (0.46 g dry weight) and growth index 3.26. However, cell growth was slower in medium added to 50 g L^{-1} sucrose with 6.13 g FW and 2.07 GI.

DISCUSSION

Auxins is required by most plant cells for division and cytokinins promote cell division[9,16]. In order to produce the final effects, the plant hormones usually interact and rarely act alone[17]. The combination between NAA or 2,4-D and BAP had successfully induced callus in some *Aquilaria* species[5,18]. Previous study mentioned that callus formation in *A. malaccensis* was significant in the combination of auxins with cytokines[5]. This research found that callus induction with high quality and more friable callus when using combination IAA or IBA or NAA with BAP than that of using single IAA, IBA or NAA.

Previous reports showed that types of explants had effect on the callus induction of many plants[19-23]. For *Aquilaria* species, callus was induced successful from some types of explants such as leaf, petiole, stem and nodal segment[18]. The result of callus induction from leaf explant in this study (88.89%) is higher in *A. sinensis*[15]. It may be possible explained that there is a difference of the medium and genotype of two experiments. Furthermore, this present study found that *in vitro* root was also suitable to induce callus with 76.67% rate and formed callus was friable. It is suggested that friable callus was required by cell suspension culture because friable callus was more suitable for dispersing the cells than compact callus[5]. Qi *et al.*[24] had used root tissue of a 2-week-old *in vitro A. sinensis* to induce callus which was the used for cell suspension culture. However, Qi *et al.*[24] did not mention of the effectiveness of callus formation and the characteristics of callus.

It is necessary to maintain callus viable for prolonged periods, especially for extensive investigation. The maintenance is performed by subculture. This is due to the growth of the cultures, depletion of components in medium

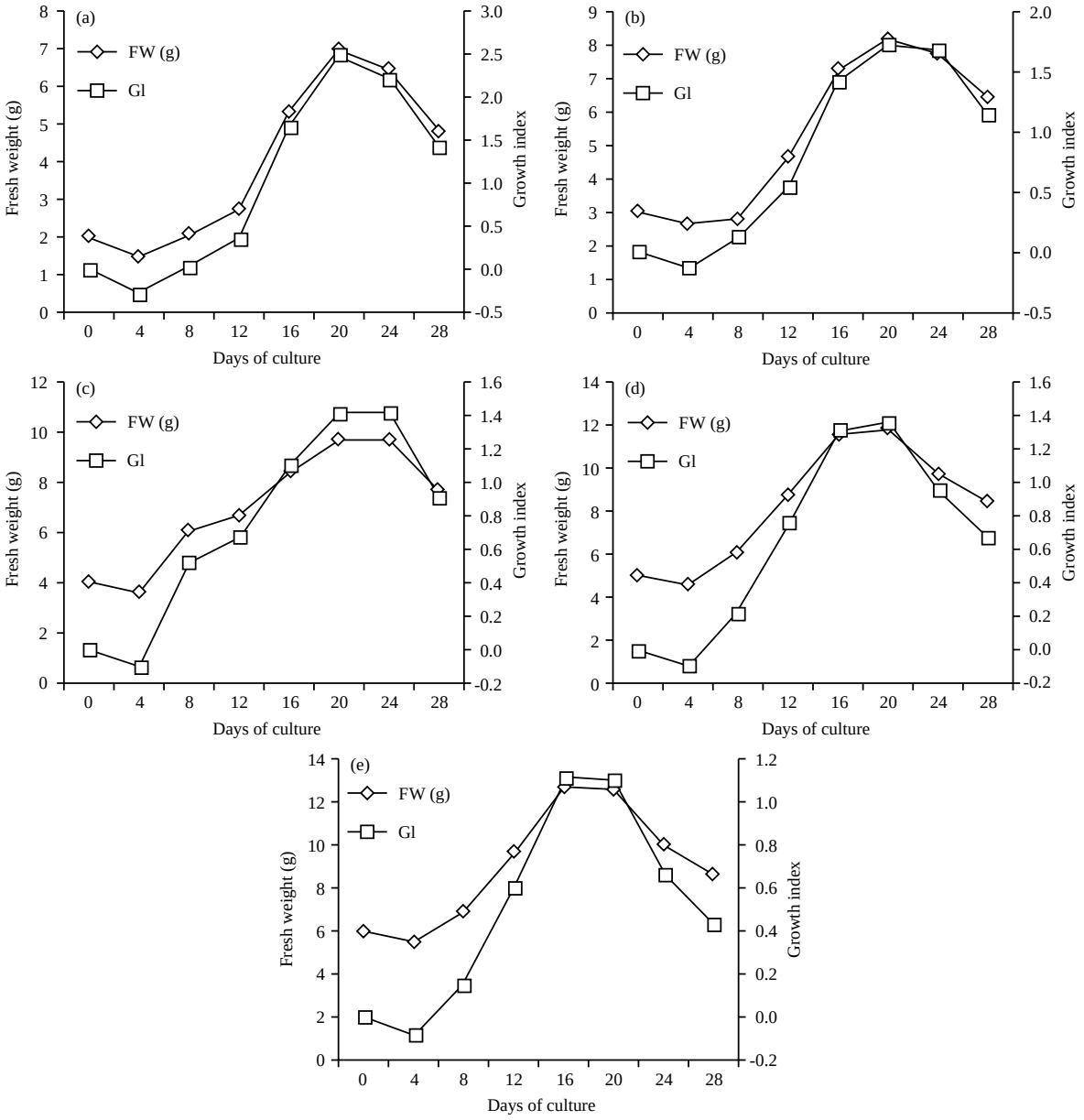

Fig. 5(a-d): Growth curve measurement of cell suspension cultures with different initial inocula sizes
(a) 2 g, (b) 3 g, (c) 4 g, (d) 5 g, (e) 6 g, Fresh weight (FW) and Growth index (GI) were determined every 4 days after inoculation

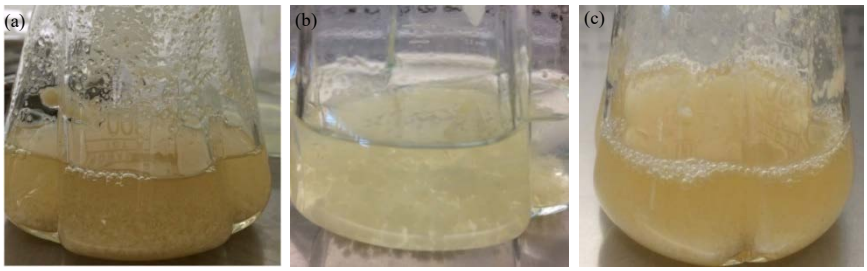

Fig. 6(a-c): Cultures were started using different callus weights
(a) 6 g, (b) 2 g, (c) Culture in MS medium supplemented 40 g L^{-1} sucrose: Photos were taken after 20 days of incubation at 26°C in the dark

(nutrient, oxygen, phytohormones) and accumulation of excretions of cultures which can lead to the death of cells[16]. Length of time for subculture is dissimilarity in different plant species. The growth of callus culture usually has 5 phases: the lag phase where cells prepare to divide, a period of exponential growth in which cell division is maximal, linear growth phase in which division slows down and cells enlarge, the cultures enter the deceleration phase where cell divisions and expansion decline and stationary period in which the number and size of the cells remain constant[9,16]. The culture should be transferred to fresh medium when it enters the end of exponential phase[16] on solid medium. For *A. rugosa* in this study, the subculture should be performed on day 28 of culture.

It is required to determine initial callus weight for the growth of cell suspension culture. A high or low density of cells is disadvantages for growth of cells. Effect of inoculum size on the growth of cell suspension culture was estimated in many plants. For instance, in ginger suspension cells when FW of inoculated cell was lower than 0.5% (w/v) ginger suspension cells proliferated slowly while the growth of cells was faster when the inoculum size was more than 2.0% but cells browned rapidly. Guo and Zhang[25] finally found that optimal inoculum size of ginger was 1%. The best inoculum for biomass cell accumulation and the amount of with anolide A production in *Withania somnifera* was found to be 10 g L^{-1} of callus FW[14]. A higher biomass in initial inoculums of *A. malaccensis* did not necessarily produce a greater amount of cell biomass[3]. Current findings showed that 2 g of friable callus FW of *A. rugosa* was sufficient for the growth of suspension cells (Fig. 5a). This can be explained that: in the same medium, there is less competition between the cells when using less callus, so the cells grew better and had more growth index. The depletion of nutrients and oxygen may have led to a faster cell death in cell cultures that started with 5 and 6 g.

In plant cell culture, sugar provides necessary carbon resource to cells. Sucrose is the most commonly used in cell culture at 2-5% (w/v)[16]. A high biomass accumulation usually was found in medium with high sucrose concentration[23]. For *Aquilaria* species, previously studies showed that the sucrose concentration was the highest in medium added 40 g L^{-1} sucrose and had significant effects on cell growth. Cell biomass of *A. malaccensis* was the highest in medium added 40 g L^{-1} sucrose[18]. This study suggested that the concentration of 40 g L^{-1} sucrose is the most suitable for accumulating cell biomass of *A. rugosa*.

CONCLUSION

A. rugosa callus was successfully induced on MS medium with combinations of auxins (IAA, IBA and NAA) and BAP which produce friable and light color callus. The most effective combination was 2 mg L^{-1} IBA and 1 mg L^{-1} BAP. Callus should be transferred to fresh medium on 28th day of culture. The establishment of cell suspension was successfully achieved with an optimal inoculum size of 2 g of friable callus FW. The growth of cell suspension was the best in MS supplemented with 40 g L^{-1}sucrose. These findings provide some basic information for further investigation using the established cell suspension culture for induction of agarwood compounds.

SIGNIFICANCE STATEMENT

This study discovered the effective protocol for establishment of callus and cell biomass in *Aquilaria rugosa*, especially inducing callus from *in vitro* stems and roots that can be beneficial for producing *in vitro* agarwood essential oil. This study will help the researchers to uncover critical areas of in vitro cultures of *A. rugosa* for agarwood production that many researchers were not able to explore. Thus, a new theory on *in-vitro* agarwood production may be arrived.

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