

An Efficient Protocol for Callus Induction in *Aquilaria malaccensis* Lam. Using Leaf Explants at Varied Concentrations of Sucrose

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Abstract *Aquilaria malaccensis* Lam. (Family: Thymelaeaceae), commonly known as agarwood, eagle wood or Gaharu is a commercially important tree species of northeast India. The *Aquilaria* tree is categorised as critically endangered in India. This species is being continuously exploited due to its precious heartwood which is the source of expensive agar oil used in the production of high grade perfumes as well as in traditional medicines. The main aim of this study was to establish a speedy callus production protocol from *Aquilaria malaccensis* which may serve as an important option for direct extraction of agar oil. For this purpose, large scale production of callus tissue is needed. An efficient callus regeneration protocol was established through leaf proliferation in *Aquilaria malaccensis* Lam. using Murashige and Skoog medium supplemented with least amount of plant growth hormones (2,4 D, NAA, Kinetin & BAP) at varied concentrations of sucrose (2-5%). Callus could be initiated in all the treatments of hormones and sugar levels; however, the best callus growth was obtained in the MS medium supplemented with BAP (0.5 mg/l) + NAA (3mg/l) giving the highest fresh (7.368g) and dry cell biomass (2.170g) at the optimum sucrose concentration (4%) after 45-60 days of incubation.

Keywords Agarwood, Callus, Growth hormones, Sucrose concentration

1. Introduction

In India, the medium sized evergreen tree *Aquilaria malaccensis* Lam. (syn. *Aquilaria agallocha* Roxb. of family Thymelaeaceae) commonly known as agarwood, eagle wood or Gaharu is confined to Eastern Himalayas up to an altitude of 1000m. The term 'Agarwood' refers to its resinous heartwood which turns aromatic and highly valuable as a result of infections by a few endophytic fungi[1]. There are fifteen species in the genus *Aquilaria* of which eight are known to produce agarwood[2]. *A. malaccensis* is chiefly distributed in 10 countries viz. Bangladesh, Bhutan, India, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore and Thailand[3]. In India, it is occurring mainly in Arunachal Pradesh, Assam, Mizoram, Manipur, Nagaland, Sikkim and West Bengal[4]. *A. malaccensis* is known to be one of the most important species of commerce and valued for production of its impregnated resinous heart wood that gives fragrance[5]. The infected parts of agarwood serve as important raw material in the production of incense,

perfumes and traditional medicines[6]. The agar wood oil or aloe wood oil, known in the east as 'agar attar' is obtained by distilling selected parts of the infected wood of *Aquilaria sp.* which has unique fragrance and high export value[7]. The agar oil traders have to sacrifice whole tree as its heart wood serves as raw materials for oil distillation. Many uninfected or less infected trees are also been destroyed by them in search of agarwood. Due to such exploitation, this tree species is now rarely found in wild habitat and considered as critically endangered in India. Consequently, it is included in IUCN red data list of the year 2011 as vulnerable and at the verge of extinction from the natural forests[8]; hence there is an urgent need to apply modern technologies for the conservation and existence of this particular species as well as for preservation of its germplasm. The application of biotechnological methods such as plant tissue culture technique may be the best alternative for the above purposes. Plant tissue culture is also important for the species which has very short dormancy period and dependent on any particular reproductive period as in case of traditional method of sexual reproduction. The *A. malaccensis* Lam. also have restricted period of seed viability[9]. The plant is generally propagated through seeds who germinate readily after maturity; however their rate of germination sharply reduces with the increase in the period of storage/decrease in its moisture contents. Fresh seeds obtained by splitting open

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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[9]. The *in-vitro* propagated medicinal plants furnish a ready source of uniform, sterile and compatible plant material for mass multiplication and help in germplasm conservation of rare, endangered and threatened medicinal plants. Taking these factors into consideration, techniques of plant tissue culture were adopted by many scientists for propagation of *Aquilaria* seedlings and to supplement the conventional methods of regeneration[10],[11]. By using tissue culture raised agarwood plantlets, growers may obtain more oleoresin than those plantlets grown from random seeds. The uniform growth with tissue culture plantlets may make the process of inducing oleoresin formation easier and volume of oleoresin more predictable. However, the proper and long-term monitoring of these seedlings is essential for their adaptation in natural conditions and for afforestation and plantation programmes for the socio-economic benefits of local populace.

Agar oil production from callus through plant tissue culture is another option which may be commercially exploited. *In-vitro* production of agar oil from callus culture of *A. agallocha* Roxb. and analysis of its chemical constituents by GC/MS have already been done successfully[12]. Chemical analysis of the oil produced from callus however showed some variations in the quality of the oil, when compared with the oil obtained by hydro distillation of fungal infected agarwood. GC/MS analysis has shown the presence of about 32 different compounds comprising of furano-monoterpenoids, acids, alcohols and aldehydes, out of which about 15 compounds could be identified. About 30% of the identified compounds showed similarity with the original agarwood oil. More such studies are needed to characterise and compare oil production capacity of calli and respective oil quality. The effect of fungal impregnation on callus and agar oil production may be another field of research which is required. Therefore, the present studies initially aimed to standardize an efficient, rapid and less expensive protocol for the production of callus tissue *in vitro* and study of salient features and growth parameters of callus tissues of *A. malaccensis* Lam., so obtained that may be utilised for essential oil production in future.

2. Materials and Methods

Leaf tissues from 3-6 month old seedlings of *A. malaccensis* Lam. were used as explants for callus induction. Explants were surface sterilized as per modified method of De[13] with a few drops of Tween-20 for 15 minute followed by thorough washing (3-4 times) with distilled water followed by with sterile distilled water. Further steps were

done under complete aseptic conditions using a laminar air flow cabinet. The explants were then treated with 70% ethanol for 1 minute, with 5-10% (v/v) sodium hypochlorite solution for 15 minutes and finally washed with sterile distilled water for 3-4 times to remove the traces of chemicals. Different hormonal combinations were tested at their varying concentrations ranging from 0-10mg/l 2,4 -Dichlorophenoxyacetic acid (2,4-D,) + 0-3mg/l Kinetin (Kn), 0.5-4mg/l 6-benzyladenine purine (BAP) + 0.5-4mg/l α -naphthalene acetic acid (NAA) and 0-10mg/l 2,4 -Dichlorophenoxyacetic acid (2,4-D) + 0.5-4mg/l 6-benzyladenine purine (BAP) for callus induction in *A. malaccensis* (Table 1). One concentration from each combination that had given the highest biomass of callus was selected for comparative studies. The surface sterilized explants were placed on MS medium[14] supplemented with different concentrations & combinations of plant growth regulators at varying sucrose concentrations (2-5% w/v) to check their effect on callus induction, growth and development[12]. The three combinations i.e. 2,4-D (2 mg/l) + Kn (0.1mg/l), BAP (0.5 mg/l) + NAA (3mg/l) and 2, 4-D (2 mg/l) + BAP (0.5mg/l) were tested and represented as combination A, combination B and combination C respectively. The pH of the medium was adjusted at 5.7 to 5.8 with the help of NaOH or HCl prior to adding 0.8% bacto agar and autoclaved at 121°C for 20 minutes. All the cultures were inoculated in 5 replicates and incubated at 25 \pm 2°C in complete dark condition. A set of flasks were also kept in light illumination (light intensity ranging from 20-40 μ E/m² /S¹) to check the effect of light on callus growth. The percentage of callus biomass on fresh weight (FW) and dry weight (DW) basis was recorded after the period of 15-30, 45-60 and 75-90 days respectively. Dry weights were taken to check the moisture contents (data not shown) in young and mature callus tissues. The morphological and microscopic features of *Aquilaria* callus were also studied as per the method prescribed by Deka[15].

3. Results and Discussion

In this paper, the emphasis was given on induction of callus in medicinally important tree species *A. malaccensis* Lam. using leaf explants. The induction and growth characteristics of *Aquilaria* callus from leaves, shoot tips and stem as explants were also reported earlier by Talukdar and Ahmed[16]. They have also obtained best callus using leaves as explants; hence, in the present studies, leaves were used for callus induction in this important tree species; however, present protocol was standardized at comparatively very reduced concentration of growth hormones. The results of our studies indicate that the optimum incubation period required is about 45-60 days in contrast to Talukdar and Ahmed[16] who obtained maximum callus in 45 days. This may be due to sub-culturing of callus in fresh medium by the investment of quite higher concentration of plant growth hormones. The hormone combination B i.e. BAP + NAA at

the concentration of 0.5 mg/l & 3 mg/l respectively was found to be the best for fast callus induction and further proliferation in the test plant species (Table1). The MS medium supplemented with hormonal combination 0.1mg/l NAA+ 0.5 mg/l BA was also found suitable and gave the best result in the formation of protocorm-like bodies (PLB) obtained from shoot tips of *Cymbidium* species[17]. We as well recorded the 4% concentration of sucrose as most favourable in MS medium as standardized earlier by Talukdar and Ahmed[16] however; callus biomass at 3% sucrose concentration was also considerably high some times highest (7.231g, 6.853g & 7.210g in combination A, B & C respectively) especially after 45-60 days of incubation. The dry cell biomass of callus was found to be proportional to their fresh cell biomass (Table1). In general, the callus growth declined in all treatments except in case of hormonal combination C, where an increase in fresh to dry cell biomass ratio was noticed.

We recorded the highest callus biomass in the MS medium supplemented with hormone combination B as 7.457g FW and 2.302g DW (Table1) at optimum concentration of sucrose (4%) after about 45-60 days of incubation. The lowest callus cell biomass was observed in hormones combination C at 2% sucrose concentration as 0.931g FW and 0.229g DW callus cell biomass after same duration of incubation.

In hormone combination A, the maximum callus biomass was obtained (7.368g FW) in 45-60 days old culture at 4% sucrose concentration whereas the lowest was recorded with 2% sucrose (1.661g FW) concentration. In combination B, the maximum callus biomass (7.457g) was obtained in 4% sucrose and the lowest was observed in 2% sucrose concentration (0.493g) after 45-60 days of incubation. Callus growth in hormone combination C depicted that 30% sucrose concentration supported the highest callus cell biomass

(7.210g) and the lowest cell biomass measured in case of 2% sucrose concentration (0.931g) after 45-60 days of incubation.

In 75-90 days incubation time, it was interesting to note that there was decrease in fresh cell biomass however, the dry cell biomass increased. The reason might be due to the fact that lignification in callus tissues might have taken place as a result of cyto-differentiation in older callus tissues and made them harder (Figure1d). It was also evident by the changes in the colour and appearance of 75-90 days old callus which looked dull and darker.

Sucrose had various effects on callus initiation and growth (Figure 3). It is the most common source of carbohydrate needed in callus induction and regeneration of plants[18]. The induction of callus occurred comparatively earlier and its growth was recorded faster in case of media treated with 4% sucrose irrespective of hormonal treatments up to 45-60 days of incubation (Figure 3C). The biomass of callus was universally proportional to the concentration of sucrose up to 4% and declined beyond it. From the present investigations, it was revealed that prolonged culturing period of callus has direct impact on its growth as weight of the callus declined sharply after 60 days of incubation. In contrast to the present findings, Talukdar and Ahmed[16] reported the highest growth of the callus on MS medium supplemented with quite higher amount of growth hormones (6mg/l 2, 4 D + 2mg/l Kn) within 30 - 45 days of culture however concentration of sucrose was optimized as 4% which support our findings. In another experiment, the highest growth of *Aquilaria agallocha* callus (547.25%) was obtained using 3% fructose as source of carbohydrate by keeping the hormonal treatment constant through leaf explants after 45 days of culture[19]; however, the maltose (60 g/l) did not supported callusing and showed the least growth rate (212.76%) in fresh cell biomass.

Table 1. Effects of different growth hormones on biomass production of *A. malaccensis* Lam. in MS medium supplemented with different concentrations of sucrose, A represents 2, 4-D (2 mg/l) + Kn (0.1mg/l); B represents BAP (0.5 mg/l) + NAA (3mg/l); C represents 2, 4-D (2 mg/l) + BAP (0.5mg/l)

Hormone Combination	Sucrose (g)	Days of culture					
		15-30		45-60		75-90	
		Fresh wt (g)	Dry wt (g)	Fresh wt (g)	Dry wt (g)	Fresh wt (g)	Dry wt (g)
A	20	0.414	0.040	1.661	0.569	1.278	0.853
	30	0.942	0.045	7.231	2.092	7.254	2.177
	40	1.281	0.237	7.368	2.170	6.952	1.805
	50	0.546	0.032	3.822	0.993	2.592	0.975
B	20	0.611	0.034	1.957	0.493	1.324	0.346
	30	1.167	0.039	6.853	1.952	5.450	0.722
	40	2.390	0.092	7.457	2.302	6.592	1.578
	50	0.478	0.144	2.598	0.887	1.894	0.384
C	20	0.494	0.018	0.931	0.229	0.971	0.244
	30	0.875	0.021	7.210	3.050	6.397	1.387
	40	0.989	0.042	6.580	1.114	6.286	0.996
	50	0.259	0.027	1.953	0.352	1.751	0.476

From the present experiment, it can be concluded that after 45-60 days of incubation, hormonal combination B and 4% sucrose were best options for rapid callus growth in *A. malaccensis* Lam. In all the treatments, 2% sucrose showed least growth (0.931gm) as compared to other sucrose concentrations (Figure 3A). In 15-30 days old callus, the highest growth was recorded in combination B (2.390g FW) followed by combination A (1.281g FW) and the lowest growth (0.259g FW) was observed in combination C in 4% sucrose (Figure 3C); but in 75-90 days old culture, callus in all treatments were declined except in hormone combination A with 3% (Figure 3B) sucrose and combination C with 2% sucrose (Figure 3A) where the negligible amount of callus were increased. No clarification could be drawn for this. At 5% sucrose concentration, reduced growth of callus was recorded however; the highest growth was noted in the hormone combination A (Figure 3D).

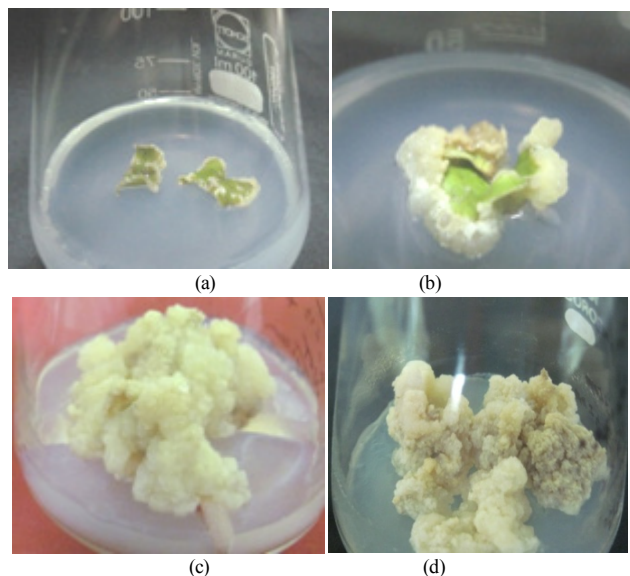


Figure 1. Stages of callus growth in *A. malaccensis* Lam. a. Initiation of callus, b&c; Growth of the callus; d. Cell death

Callus tissues of *A. malaccensis* which were grown and maintained under dark become whitish or creamish yellow in colour (Figure 1 a-c). They usually turned green when transferred under light illuminations (intensity $20-40\mu\text{E}/\text{m}^2/\text{S}^1$) due to development of chloroplastids. Aceto-carmin staining of young and matured callus tissues revealed the presence of embryogenic cells [15] which appeared crimson red in colour however non-embryogenic cells appeared unstained, distorted and irregular in shape or with lighter shade due to lack of protoplasm. Squash preparation of the callus tissue of *A. malaccensis* showed that the cellular composition was heterogeneous ranging from small cells with dense cytoplasm to large cells with vacuolated cytoplasm in case of young or friable callus. The friable callus cells were loose, with entire margin, full of

protoplasm and mostly globular in shape with average diameter of $31.5\mu\text{m}$. The other shapes like oval, slightly elongated, beaked or comma shaped ranging between $28-45\mu\text{m}$ in length and $18-35\mu\text{m}$ in breath were also observed (Figure 2).

Oil extraction from callus of agarwood had successfully been done earlier [12] and reported that the oil has about 30% similarities with that of agar oil extracted from natural wood. In *Taxus baccata* L. Washingtonii, the valuable "Taxol", an anticancer agent is being extracted from callus tissues [20]. As the *A. malaccensis* is valued for agar oil, the mass callus production shows great promises to extract essential oil directly in future without sacrificing the whole tree.

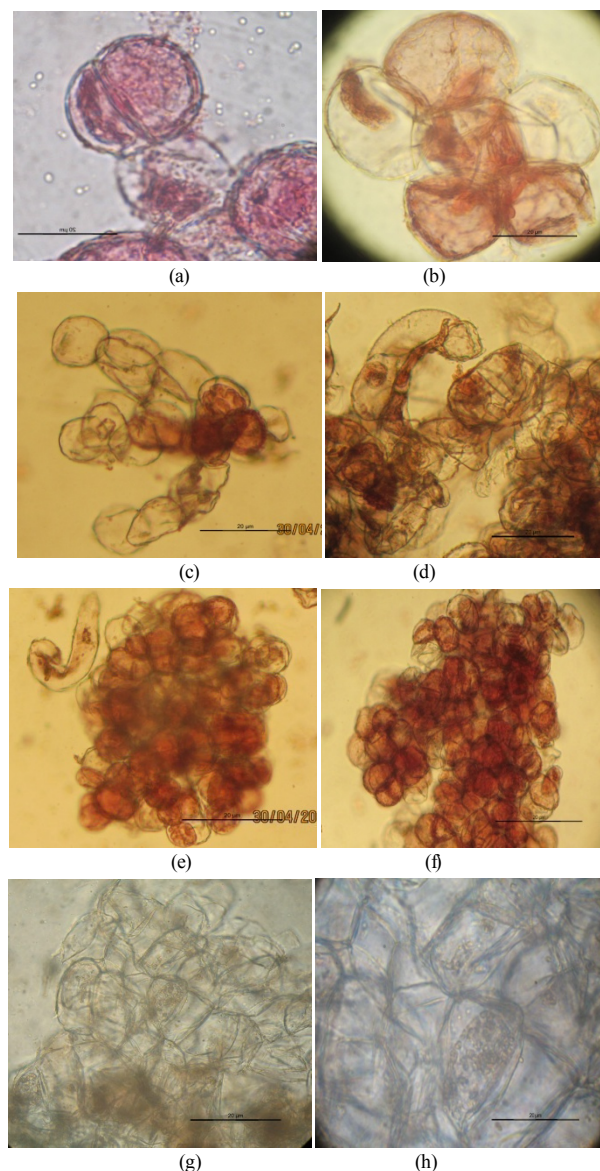
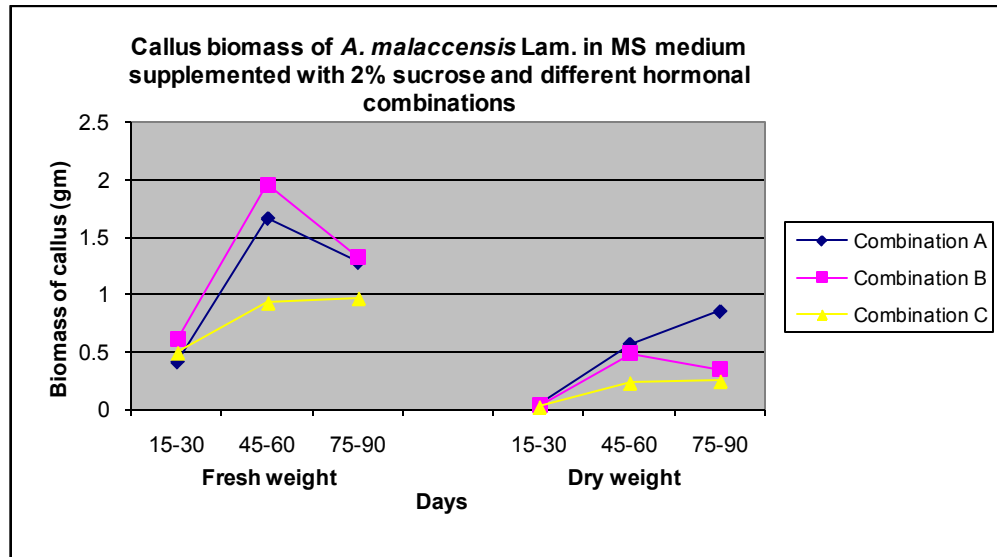
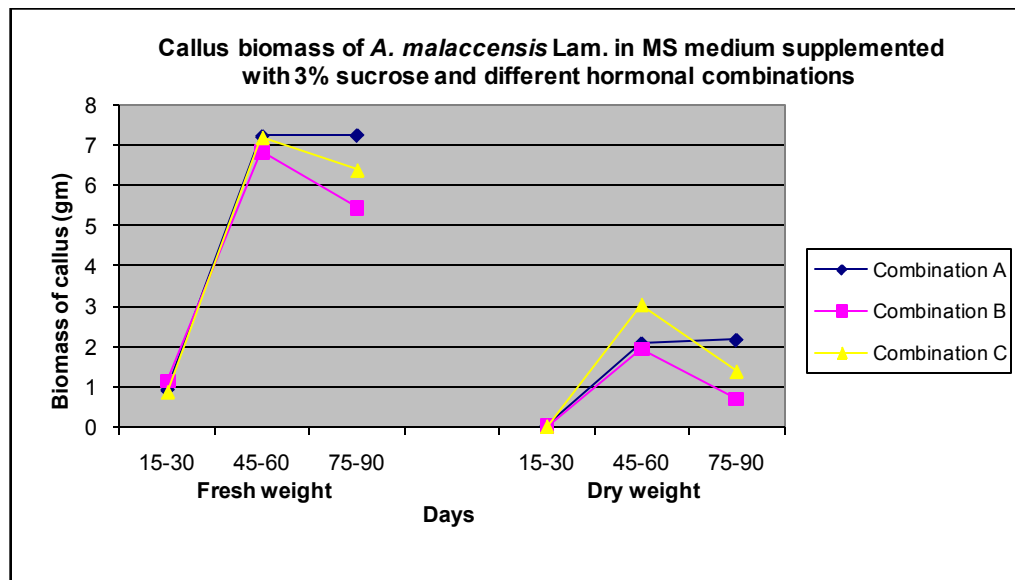


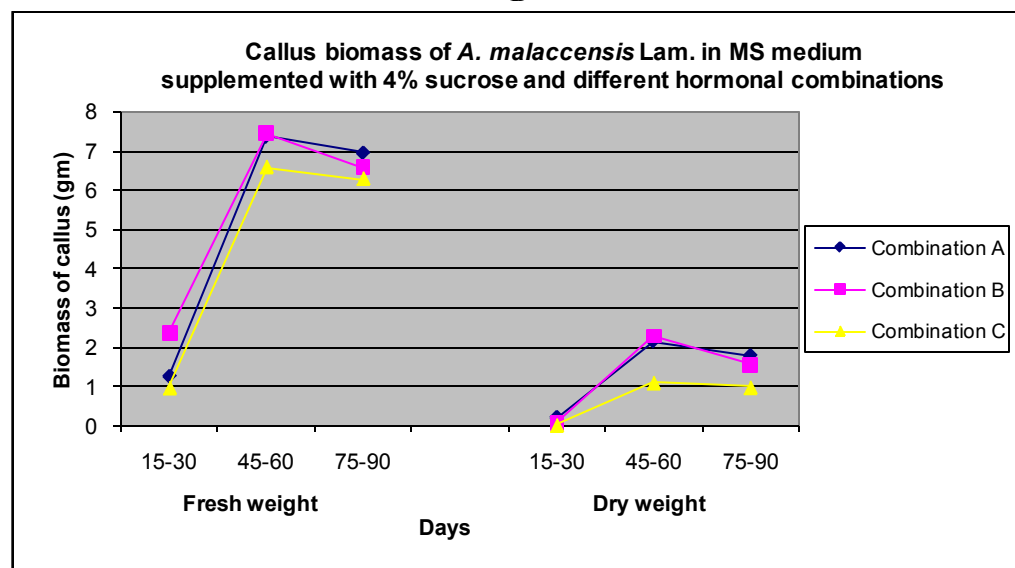
Figure 2. Callus tissues of *A. malaccensis* Lam. a-f. Embryogenic cells of various shapes; g-h. Non-embryogenic cells; Scale Bar: a-h = $20\mu\text{m}$



A



B



C

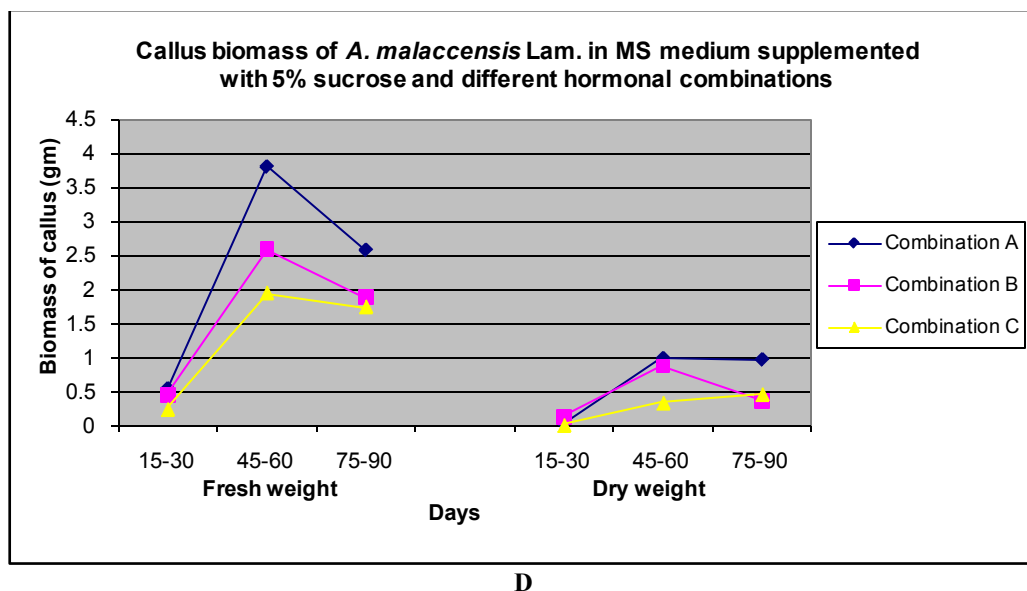


Figure 3. Callus biomass of *A. malaccensis* Lam. in MS medium with different hormonal combinations. A. 2% sucrose concentration; B. 3% sucrose concentration C. 4% sucrose concentration, D. 5% sucrose concentration

4. Conclusions

From the present investigations, a reproducible, standard and efficient tissue culture protocol for callus production using leaf explants in *A. malaccensis* Lam. has been defined which has importance from point of view of use of very less hormonal concentrations. MS medium supplemented with BAP (0.5 mg/l) + NAA (3mg/l) at 4% sucrose concentration was found the best for obtaining maximum callus biomass in agar species. Overall, combination A was found more suitable in terms of callus biomass at 3-5% sucrose concentrations as compare to others. As the tree is economically important for preparation of perfumes, incense and traditional medicines, the present investigations may be helpful for large scale production of callus biomass and to oil traders for synthesis of agar oil from it. Another interesting area of research may be to investigate effect of introduction of endophytic fungi in it and its effect on agar oil quality and quantity analysis.

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