Callus Production and Resin Induction of Agarwood (*Aquilaria crassna)*

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Abstract
Agarwood is widely known for its fragrant resinous heartwood, refers to species in following four genera: Gyrinops, Aetoxylon, Gongystylis and more commonly, Aquilaria in the family Thymelaeaceae. This species is continuously exploited due to its precious resinous 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 callus production protocol from *Aquilaria crassna* which may serve as an important option for direct extraction of agar oil through *in-vitro* culture. Large scale production of callus tissue is needed for this purpose. An efficient callus regeneration protocol was established through leaf proliferation in *Aquilaria crassna* using Murashige and Skoog (MS) medium supplemented with plant growth substances: α Napthalene acetic acid (NAA) and 6-Benzyl aminopurine (BAP). The leaf ex-plants were collected from potted mother plants that were maintained inside shade house. Experiments were carried out to find out the best surface sterilization method for ex-plants and best hormone combination for callus induction. The best Clorox concentration for surface sterilization was applied for ex-plants with different level of NAA and BAP combinations.MS medium was used for callus induction. The best callus growth was obtained in the MS medium supplemented with BAP (0.5 mg/l) + NAA (3mg/l) giving the highest fresh (7.368 g) and dry cell biomass (1.203 g). Induced calli were transferred to MS medium containing Salicylic acid, Ferric chloride and Formic acid to check whether artificial resin induction could be obtained. Two weeks after, the calli grown on different chemicals were subjected to Thin Layer Chromatography (TLC) and compared with standard Agarwood resin as a control. The used levels of chemicals were found to be ineffective for resin induction in callus cultures.

Key words: Agarwood, *Aquilaria crassna, Callusculture,* Resin Induction
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Introduction

Agarwood is dark resinous heartwood that forms in *Aquilaria, Gyrinops, Aetoxylon* and *Gonystyis* trees of family Thymelaeaceae. Nine species out of seventeen recorded in *Aquilaria* and two species out of eight recorded in *Gyrinops* species are identified with the ability of producing agarwood (Chetpattananondh, 2012). The resin embedded wood is commonly called as agarwood, and found in countries like Myanmar, Vietnam, Cambodia, Malayasia, Indonesia, Thailand, South Korea, Philippines, Laos and Japan. Resin extracted from *Aquilaria* species has its unique fragrance and therefore widely used as an ingredient in traditional medicine and for the production of superior quality perfumes, incenses, and in aroma therapy (Barden *et al.*, 2000). Production of fragrant resin is associated with wounding and fungal infection, possibly assisted by insects. As a mode of self-defense mechanism, the tree produces a resinous material high in volatile organic compounds that aids in suppressing or retarding the growth of fungus. Various fungi are associated with agarwood formation although it is still not completely clear which ones induce the plant to form resin. The infected part of the tree separated and oil processed from it.

Due to the high economic value in perfumery and other industries, over exploitation of resin producing species, from natural habitats has been reported from many countries. Therefore, they are classified as endangered species in the international trade (Jensen, 2007). Under the natural environment, development of resinous heartwood takes long time, but due to increasing international demand for resin resulted in over exploitation and even fully mature trees yield very low amount of resin.

Therefore, development of large scale resin production system from Agarwood is of high commercial and conservational importance. An efficient and effective resin production method in short period of time helps to conserve the agarwood plants in natural forests and maximize the profit. This study was planned with the objective of developing an *in vitro* protocol for mass production of Agarwood (*Aquilaria crassna*) calli and resin induction through calli using different chemicals.

Materials and Methods

Leaf tissues from 3-6 month old seedlings of *A. crassna* were used as explants for callus induction. Explants were surface sterilized with different concentrations of Clorox (20% Clorox

for 15 minutes, 25% Clorox for 15 minutes and 25% Clorox for 20 minutes) and few drops of Tween-20 for 15- 20 minutes followed by thorough washing (3-4 times) with distilled water to find out the best concentration and exposure time period for surface sterilization. The explants were then treated with 70% ethanol for 1 minute, finally washed with sterile distilled water for 3-4 times to remove the traces of chemicals.

Experiment was arranged as a Completely Randermized Design and non contamination percentage was recorded. The surface sterilized explants were cultured in MS medium supplemented with different concentrations and combinations of plant growth regulators to check their effect on callus induction, growth and development. Different hormonal combinations were tested at varying concentrations ranging from 0.5-3 mg/l α-naphthalene acetic acid (NAA) with 0.5 mg/l 6-benzylamino purine (BAP) for callus induction. There were five replicates from each treatment. 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% agar and autoclaved at 121ºC for 20 minutes.

Cultures were incubated at 25º± 2ºC in complete dark for 2 weeks. After two weeks, the cultures were transferred to light. The callus biomass on fresh weight (FW) basis and callus health as assessed by the appearance of callus were recorded twice; after 15-30 and 45-60 days respectively. Established calli were transferred to the MS medium supplemented with Salicylic acid, $FeCl_3$, and Formic acid at 1,3 and 5 ppm concentrations respectively, for the induction of resin formation. According to Robert *et al.* (2010) these chemicals and concentrations successful in resin induction in field grown plants. Two weeks after sub-culturing, calli were prepared for Thin Layer Chromatography (TLC). R_f values were calculated using TLC plates and compered with the standered agarwood resin samples for identification.

Results and Discussion

The emphasis was given on induction of callus in *A. crassna* using leaf explants. The induction and growth characteristics of *Aquilaria* callus from leaves shoot tips and stem explants were reported earlier by Talukdar and Ahmed (2002). They have obtained best callus using leaf explants; hence leaves were used for callus induction in the present study. However present protocol was standardized at comparatively lower NAA and BAP concentrations compared to the levels used by Talukdar and Ahmed (2002).

Selection of Best Surface Sterilization Procedure for Ex-Plants

In this experiment, three different concentrations of Clorox with different exposure time periods were tested. After 21 days of incubation, percentages of contaminated leaf discs were observed. Fungal contamination was observed as hyphal growth from the explants and bacterial contamination was identified by observing colonies, seen as watery or slimy buildups on the agar.

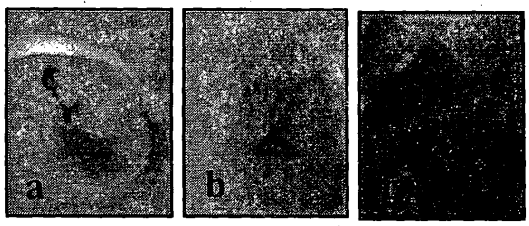

Figure 1: Effect of different sterilization methods on appearance of leaf discs: a. 20% Clorox for 15 minutes, b. 25% Clorox for 15 minutes and c. 25% Clorox for 20 minutes

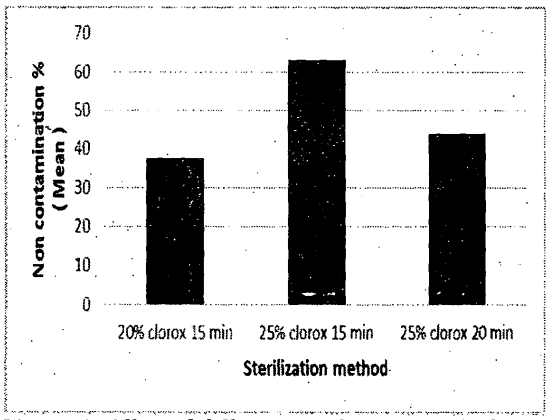

Figure 2: Effect of different sterilization methods on non-contamination percentage of leaf discs

Results revealed that, 25 % Clorox for 15 minutes were the best treatment for leaf discs establishment with (64%) of non-contaminated leaf disc cultures (Figure 2). Although, treatment three has low non-contamination percentage than treatment one, it caused bleaching of ex-plants (Figure 1c).

Optimum NAA and BAP combination for Callus Induction of Aquilaria leaf callus explants

Different NAA concentrations; 0.5-3 mg/L were tested with 0.5 mg/L BAP for callus induction from *Aquilaria* leaf explants in MS medium. The

results indicated that the optimum incubation period required for successful callus induction was 30-45 days. The most effective BAP + NAA concentration for the fast callus induction and further proliferation in *Aquilaria* leaf disc explants was 0.5 mg/l BAP with 3 mg/l NAA respectively (Table 1). In this hormone combination, the maximum callus biomass was obtained (7.368g FW) in 45-60 days and the lowest cell biomass (0.931g FW) was observed in 0.5 mg/l BAP+ 0.5 mg/l NAA after 45-60 days of incubation.

Table 1: Effect of different growth regulator combinations for fresh and dry weight of callus

NAA+ BAP (mg/l)	Callus weight			
	15-30 (days)		45-60 (days)	
	FW(g)	DW(g)		FW(g) DW(g)
0.5 + 0.5	0.321	0.023	1.213	0.046
1.0+ 0.5	0.623	0.034	1.821	0.052
2.0+ 0.5	1.223	0.049	5.371	0.752
3.0+ 0.5	2.235	0.092		7.221 1.203

FW- Fresh weight DW- Dry weight

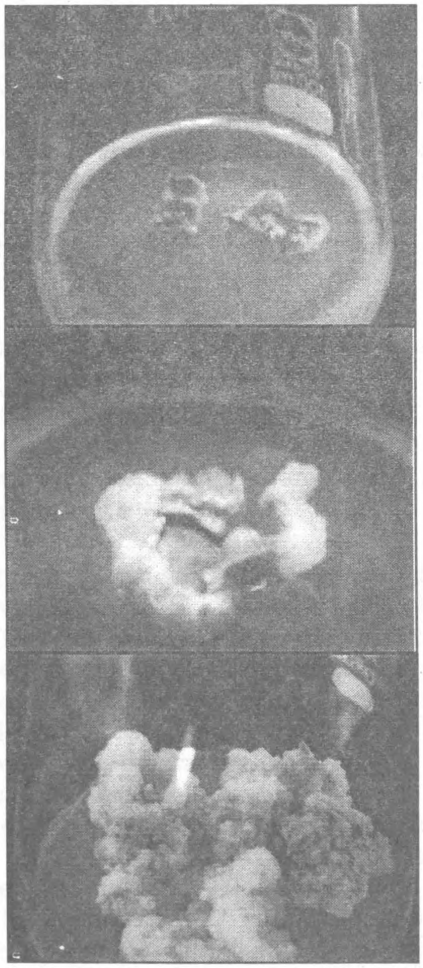

Figure 3: Stages of callus growth in MS medium with BAP 0.5 mg/l and NAA 3 mg/l

In the present experiment, *A. crassna* leaf explant cultures incubated with BAP+ NAA at a rate of 0.5 mg/l BAP with 2 or 3 mg/l NAA provided higher callus mass at both culture durations: 15 – 30 days and 45 – 60 days. Accordingly, it can be concluded that above BAP + NAA concentrations and after 45-60 days of incubation, rapid and healthy callus growth can be obtained from *A. crassna* leaf explants.

TLC analysis does not indicate the presence of resins in callus extractions, indicating either the used levels of chemicals may not be sufficient to induce resin biosynthesis or the callus cells needed to be more matured to be able to produce resinous material. The applied levels of inductive chemicals are successfully worked in inducing resin in field grown trees. Therefore, it can be concluded that further studies with a range of concentrations of resin inductive chemicals as well as callus cultures at different maturity are needed to investigate the possibilities of *in-vitro* resin biosynthesis from *A. crassna* leaf callus cultures.

Successful agarwood oil extraction from callus cultures was reported earlier (Talukdar *et al.*, 2002). However, it has been found that the composition of in-vitro produced oil has only about 30% similarity to agar oil extracted from natural wood. As the *A. crassna* is commercially valued for agar oil, the ability of mass callus production through leaf explant culture shows great promise to extract essential oils *in-vitro* once the protocol is finalized, without sacrificing the whole tree.

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