ISSN 1996-3351

Asian Journal of Biological Sciences

Knowledgia
SCIENTIFIC
A Place to Publish Outstanding Research

http://knowledgiascientific.com

Asian Journal of Biological Sciences 6 (2): 96-105, 2013
ISSN 1996-3351 / DOI: 10.3923/ajbs.2013.96.105
© 2013 Knowledgia Review, Malaysia

Effect of Culture Media and Growth Hormones on Callus Induction in *Aquilaria malaccensis* Lam., a Medicinally and Commercially Important Tree Species of North East India

Moitreyee Saikia, Karuna Shrivastava and S. Sureshkumar Singh
Laboratory of Biotechnology and Plant-Microbe Interaction, Department of Forestry, North Eastern Regional Institute of Science and Technology, Nirjuli-791109, Arunachal Pradesh, India

Corresponding Author: Karuna Shrivastava, Laboratory of Biotechnology and Plant-Microbe Interaction, Department of Forestry, North Eastern Regional Institute of Science and Technology, Nirjuli-791109, Arunachal Pradesh, India

ABSTRACT

The use of *in vitro* culture technique has growing significance in conservation of plant germplasm and in securing valuable products of medicinal and commercial importance. *Aquilaria malaccensis* Lam., an economically important tree species of North East India is valued for its highly prized wood and agar oil. Callus induction was investigated using young leaf and nodal explants of this species on modified Murashige and Skoog (MS) and Woody Plant Medium (WPM) supplemented with different growth hormones. We found MS medium more suitable for induction and maintenance of callus when supplemented with growth hormones at high auxin and low cytokinin ratio. The hormonal combination of 2,4 dichlorophenoxyacetic acid (2,4D)+kinetin was recorded as most appropriate for high percentage of callus induction (70-73%), fast growth rate of viable and healthy callus. Rhizogenesis and embryogenesis from callus tissues of *Aquilaria malaccensis* could be accomphished. In laboratory conditions, large scale and rapid callus production protocol has been established which may be utilized for *in-vitro* biosynthesis of biochemical principles of commercial importance. This may help in reducing uncontrolled overexploitation of this endangered tree species while giving an important opportunity to conserve the natural population in the wild habitats of the region in particular and other habitats in general.

Key words: *Aquilaria malaccensis*, callus, plant growth regulators, leaf explant, North East India

INTRODUCTION

The Agarwood tree (*Aquilaria malaccensis* Lam.) belongs to the family Thymelaeaceae is a commercially important and critically endangered forest tree species of northeast India. The plants are continuously exploited due to its aromatic heartwood to be used for the extraction of most precious agar oil. This species is commonly known as agarwood and has various local names such as Aloe wood, Eagle wood, Sashi, Gaharu, Agaru etc. Natural population of the species mainly occurs in the hills and forests of Arunachal Pradesh, Nagaland, Manipur, Mizoram and Tripura as well as in West Bengal (Palit, 1996). Of the fifteen species of *Aquilaria* reported across the world, eight are known to produce agarwood (Kosmiatin *et al.*, 2005). The black resinous heartwood or 'agaru' and agar oil is the most preferred perfumery raw materials obtained by the distillation of the infected wood of *Aquilaria* spp. Agarwood is valued not only for its aromatic properties but also used as a medicinal plant. It is used as stimulant tonic, diuretic, in the treatment of asthma, colic,

chest congestion, diarrhoea, body pain, rheumatism, etc. (Kim *et al.*, 1997; Bhuiyan *et al.*, 2009; Burkill, 1966; Chakrabarty *et al.*, 1994; Barden *et al.*, 2000). The plant is also reported to possess remarkable anti-cancer activity (Gupta *et al.*, 1995). Naturally, *Aquilaria* species regenerate through seeds but high mortality and low survival rate are the major constraints leading to its declined population in the wild. The seeds of the plant have physiological limitations such as short dormancy period, rapid loss of moisture and rapid decline in seed weight which causes poor seed viability prior to germination. Moreover, the seeds exhibit a very short life of few days to a week only and rapid loss of seeds moisture during the first few hours to few days leads to rapid loss of viability; adversely affecting the germination rate. Furthermore, insect attack and pests infestation often inhibits the growth of the seedlings and saplings in the early phase of establishment (Okugawa *et al.*, 1996). Because of the above facts coupled with unauthorised overexploitation of this tree for commercial extraction of agar oil and wood, the population status becomes rare in the wild habitats and declared as critically endangered species from conservation point of view.

Recently, there was noticeable decrease in the population of *A. malaccensis* in natural forests of north east India and mainly due to unlimited harvesting of its mature trees for agarwood trade. As a result, the species has been included in International Union for Conservation of Nature (IUCN) red data list as vulnerable and reported to be at the verge of extinction from the natural forests (www.iucnredlist.org). Therefore, there is an urgent need to develop suitable regeneration strategies to supplement its natural regeneration and conservation programmes with sustainable exploitation (Saikia and Khan, 2013). Application of biotechnological tools and techniques, particularly plant tissue culture in regeneration, mass propagation and *in-vitro* conservation of rare and endangered medicinal and economically important plants have shown a rapid success in the recent past (Rai, 2010; Verma *et al.*, 2012; Najar *et al.*, 2012). *In-vitro* plant regeneration technique has become a popular tool and useful method to overcome such problems if applied skilfully. By using tissue cultured agarwood plantlets, growers obtain more oleoresin than by growing from seeds. The uniform growth rate and enhanced physiological function of tissue culture raised plantlets may reveal the mechanism of inducing oleoresin production and predictable volume of oleoresin. Hence, the present research investigations propose to develop a reproducible and fast protocol for large scale production of callus of *A. malaccensis* which could be utilized for organogenesis and direct oil production purposes of this commercially important tree species.

MATERIALS AND METHODS

Plant materials: The seeds of agarwood tree (*Aquilaria malaccensis* Lam.) were collected twice in July 2009 and 2010 from the wild natural habitats of Assam and Arunachal Pradesh. Freshly collected seeds were used for raising the seedlings in the Nursery of the Department of Forestry, NERIST and same were used as source of explants for the present studies. The leaves and nodal explants from 3-6 months old seedlings were used for induction of callus culture under controlled laboratory and environmental conditions.

Establishment of *Aquilaria* calli: Fresh leaves and nodal explants were first rinsed by treating with a few drops of Tween-20 in water for 15 min followed by thorough washing with distilled water and finally again washed with sterile distilled water (3-4 times). These surface sterilized explants were used for all the culture studies under aseptic conditions. The explants were taken into laminar airflow, treated with ethanol (70%) for 1 min, with sodium hypochlorite solution (5-10% v/v) for 10-15 min and washed with sterile distilled water for 3-4 times to remove all traces

Table 1: List of various hormonal treatments tested for callus induction from leaf and nodal explants of *Aquilaria malaccensis*

Treatment	Combination of hormones (mg L^{-1})			Treatment	Combination of hormones (mg L^{-1})		
Leaf explant							
T1	MS Basal	0	-	T26	MS+CW (mL L^{-1})	5	-
T2	MS+2,4D	0.5	-	T27	MS+CW(mL L^{-1})	10	-
T3	MS+2,4D	1	-	T28	MS+CW(mL L^{-1})	15	-
T4	MS+2,4D	2	-	T29	MS+CW(mL L^{-1})	20	-
T5	MS+2,4D	3	-	T30	WPM Basal	0	0
T6	MS+2,4D	4	-	T31	WPM+NAA+BAP	0.5	0.5
T7	MS+2,4D	6	-	T32	WPM+NAA+BAP	1	0.5
T8	MS+NAA+BAP	0.5	0.5	T33	WPM+NAA+BAP	2.5	0.5
T9	MS+NAA+BAP	1	0.5	T34	WPM+2,4D+Kinetin	1	0.5
T10	MS+NAA+BAP	2	0.5	T35	WPM+2,4D+Kinetin	2	0.5
T11	MS+NAA+BAP	3	0.5	T36	WPM+2,4D+Kinetin	4	0.5
T12	MS+2,4D+Kn	2	0.1	**Nodal explant**			
T13	MS+2,4D+Kn	2	0.5				
T14	MS+2,4D+Kn	2	1	T37	MS Basal	0	0
T15	MS+2,4D+Kn	4	1	T38	MS+2,4D	1	-
T16	MS+2,4D+Kn	6	1	T39	MS+2,4D	2	-
T17	MS+2,4D+Kn	8	1	T40	MS+2,4D	4	-
T18	MS+2,4D+Kn	2	2	T41	MS+2,4D+Kinetin	2	0.1
T19	MS+2,4D+Kn	4	2	T42	MS+2,4D+Kinetin	2	0.5
T20	MS+2,4D+Kn	6	2	T43	MS+2,4D+Kinetin	2	1
T21	MS+2,4D+Kn	8	2	T44	MS+NAA+BAP	0.5	1
T22	MS+2,4D+Kn	2	3	T45	MS+NAA+BAP	0.5	2
T23	MS+2,4D+Kn	4	3	T46	MS+NAA+BAP	0.5	3
T24	MS+2,4D+Kn	6	3	T47	MS+NAA+BAP	1	0.5
T25	MS+2,4D+Kn	8	3	T48	MS+NAA+BAP	2	0.5

of chemicals. The explants were then aseptically placed on the surface of Murashige and Skoog (MS) medium and Woody Plant Medium (WPM) supplemented with different concentrations of phytoregulators in 3% (w/v) of sucrose concentration (Murashige and Skoog, 1962; Lloyd and McCown, 1981). The pH of the medium was adjusted to 5.7-5.8 with 0.1 N NaOH or HCl prior to adding 0.8% agar and autoclaved at 121°C for 20 min. MS medium supplemented with 2, 4-Dichlorophenoxyacetic acid (2,4-D) at 0-8 mg L^{-1}, α-Naphthalene Acetic Acid (NAA), at 0-4 mg L^{-1}, Kinetin and 6-Benzyladenine Purine (BAP) at 0-4 mg L^{-1} alone or in combinations were used for callus induction. Coconut Water (CW) was also included in MS medium for the study of its effect on callus initiation and growth. Detail of various hormonal treatments (T1 to T48), their concentrations and combinations are given in Table 1. The plain WPM (T1) and basal MS media (T30 and T37) without any growth hormone were treated as control for leaf and nodal explants, respectively. WPM was not used in case of nodal explants. All the cultures were raised in wide mouth conical flasks or test tubes and maintained in warm white florescent light of 20-40 µE m^{-2} $/S^{1}$ intensity for 16 h photoperiod at 25±2°C temperature. Callus induction percentage was calculated for each hormonal treatment with the following formula:

$$\text{Callus induction (\%)} = \frac{\text{Total No. of explants with callus induction}}{\text{Total No. of explants inoculated for each treatment}} \times 100$$

The callus growth rate was recorded per day basis of callus induction and graded by comparison in four categories as very good (++++), good (+++), poor (++) and very poor (+). The callus morphology such as color, appearance, texture, pigmentation etc. were noted as per hormone treatments. The data so obtained were tested statistically using ANOVA with IBM-SPSS 20.0 and mean differences were tested using Duncan's multiple range test at $p<0.05$ level.

Regeneration studies: The fresh callus tissues were used and tested for regeneration of shoots and roots. Induced calli were placed in embryogenic and organogenic callus proliferation media supplemented with different concentration of plant growth regulators including BAP (1-5 mg L^{-1}), NAA (0.5-2 mg L^{-1}) alone or in combination of BAP with NAA (0.1-1 mg L^{-1}) or Indole-3-Acetic Acid (IAA) (0.1-1 mg L^{-1}) and/or without any hormone for regeneration through embryogenesis and organogenesis. Twenty replicates were taken for each treatment and medium without hormone was treated as control.

RESULTS AND DISCUSSION

The callus could be induced using callus induction media (MS and WPM) from both young fresh leaves as well as nodal explants. Hormone played important role in callus induction rate with varying results in different hormonal treatments and combinations. Rapid callus induction was obtained in case of leaf explants as compare to stem nodal explants irrespective of culture media used or growth regulators treated. Earlier, the effect of varied sucrose concentrations on callus induction was investigated in *Aquilaria malaccensis* Lam. using leaf explants (Saikia *et al.*, 2012). Maximum callus biomass was obtained in MS medium supplemented with BAP (0.5 mg L^{-1})+NAA (3 mg L^{-1}) at 4% sucrose concentration. The induction and growth characteristics of callus of *Aquilaria malaccensis* had also been carried out using leaves, stems and shoot tips as explants where best results were obtained from leaves (Talukdar and Ahmed, 2001). In the present investigations, fastest initiation of callus was recorded on 12th day using MS medium with leaves as explants. The hormonal treatments T13 (MS+2,4-D 2 mg L^{-1}+kinetin 0.5 mg L^{-1}) and T11 (MS+NAA 3 mg L^{-1}+BAP 0.5 mg L^{-1}) could induce callus faster with an average period of 14 and 15 days, respectively (Table 1, 2). On the contrary, as in WPM, callus induction took long time i.e., 28 days with an average period of 31 days after incubation for all replicates. The highest callus multiplication rate (+++) was observed in treatment number T33 (WPM+NAA 2.5 mg L^{-1}+BAP 0.5 mg L^{-1}). The maximum callus induction percentage (73%) was recorded through leaf explants in case of treatment number T12 (MS+2,4-D 2 mg L^{-1}+kinetin 0.1 mg L^{-1}) (Table 1) followed by treatments T13 and T11 with 60% each (Table 2). MS medium supplemented with CW showed slow and poor callus initiation but it showed fast growth with profuse calli when transferred into the fresh medium. CW is known to serve as a source of natural growth regulators influencing *in-vitro* growth of plant tissues (George, 1993). However, in the present studies CW did not influence early initiation of callus but it had enhanced further callus multiplication.

In case of nodal explants, callus induction took place on 14th day with an average of 17 days in treatment number T41 (MS+2,4-D 2 mg L^{-1}+kinetin 0.1 mg L^{-1}) using MS nutrient medium (Table 2). The best induction percentage (70%) was recorded in hormonal treatment T41 followed by T42 (MS+2,4-D 4 mg L^{-1}+kinetin 0.5 mg L^{-1}) and T48 (MS+NAA 2 mg L^{-1}+BAP 0.5 mg L^{-1}) with 50 % each (Table 2).

Table 2: Effects of different concentration of hormone (s) alone and/or in combination for callus induction and callus growth from leaf and nodal explants of *Aquilaria malaccensis*

Hormone treatment	Callus indnction (%)	Rate of callus growth	Hormone treatment	Callus induction (%)	Rate of callus growth
Leaf explants					
T12	73_a	++++	T32	30_e	++
T11	60_b	++++	T21	25_f	++
T13	60_b	+++	T25/T28	20_f	+
T16/T23/T34	50_c	++	T1-T8/T26/T27/T30	0	-
T19	50_c	+++	**Nodal explants**		
T33	50_c	+++	T41	70_a	++++
T14	47_d	+++	T42	50_b	+++
T15	47_d	++	T48	50_b	++
T18	42_d	+	T39	40_c	+
T5/ T24	40_d	+	T43	40_c	++
T29	40_d	++	T46	40_c	+++
T9/T20/T35	33_e	+++	T36	33_c	++
T10/ T17/ T31	33_e	++	T40/T47	30_c	+
T4/ T22	30_e	+	T37/T38/T44/T45	0	-

Means followed by same letter are not significantly different at $p<0.05$ level, ++++: Very good, +++: Good, ++: Poor, +: Very poor, -: No callus formation

The treatments with hormonal combinations MS+2, 4-D 2 mg L^{-1}+kinetin 0.1 mg L^{-1} (T12 and T41) may be considered as optimum for callus induction and growth for agarwood species. It provided highest callus induction percentage (73% and 70%; r value= 0.45 and 0.40, respectively at 5% significant level) in both leaf and nodal explants. The minimum induction percentage were recorded (20%) in T25 and T28 (r-value = 0.35 at 5% significant level) in leaf explants. In case of nodal explants, only 30% callus induction was recorded in treatment numbers T40 and T47 (r value = 0.36 at 5% significant level).

When the observation on callus induction was made for all treatments on 18th day after inoculation on the nutrient media, it was found that as maximum as 90% callus induction occurred in T13 and T11 with that of leaf explants and T41 with that of nodal explants (Fig. 1a and b). The minimum callus induction percentage were recorded to be 11.5% in T25 for leaf explants and T40 for nodal explants respectively (Fig. 1a, b).

In nodal explants, multiple shoots were initiated in MS medium supplemented with low concentration of auxin (NAA, 0-0.5 mg L^{-1}) and high concentration of cytokinin (BAP, 1-2 mg L^{-1}). Induction of multiple shoots was stopped when the explants were grown in the same medium with higher cytokinin concentration (<2.5 mg L^{-1} BAP) with or without NAA which increased initiation and formation of callus at the base of the explants. Fully grown callus could be maintained in both induction and basal MS medium though induction medium supported longer period of healthy, white and friable callus at faster multiplication rate. No notable callus formed in the MS and WPM media when 2, 4-D, NAA or kinetin was used separately, otherwise callus initiated lately in tiny amount using both leaf and nodal explants. No explants responded for callus initiation on basal MS and WPM media which were used as controls.

Combinations of both 2,4-D and Kinetin for initiation of callus in some woody plant species viz. *Phoenix dactylifera, Sesamum indicum* and *Ceratozamia hildae* have been reported by several workers (Omar and Novak, 1990; Rao and Vaidyanath, 1997; Litz *et al.*, 1995). MS medium supplemented with lower levels of 2,4-D (2 mg L^{-1}) and Kinetin (0.5 mg L^{-1}) was recorded to initiate

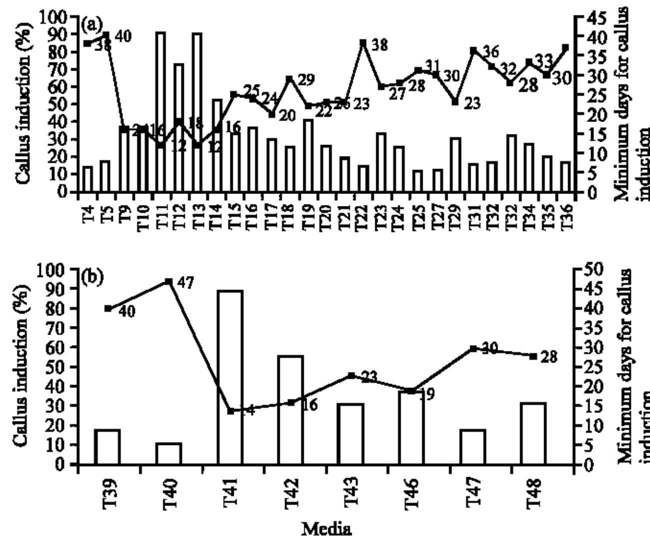

Fig. 1(a-b): The values of percent callus induction and minimum days required from (a) Leaf and (b) Nodal explants of *A. malaccensis* in different hormonal treatments as calculated on 18th day after inoculation

the callus in *Havea brasiliensis* (Kumari *et al*., 1999). Our studies report fast callus induction and multiplication rate in *Aquilaria* species at very low hormonal (MS+2,4D 2 mg L^{-1}+Kinetin 0.1 mg L^{-1}) and sucrose (3%) concentrations. Enhanced rate of callusing in *Aquilaria* species has been reported earlier using various carbohydrate sources at different concentrations (Talukdar and Ahmed, 2001). The maximum callus was obtained only after 30- 45 days when comparatively higher amount of growth regulators and sucrose (4%) were used (MS medium+2, 4-D, 6 mg L^{-1}+Kinetin, 2 mg L^{-1}). Similarly, highest growth of *Aquilaria agallocha* callus was obtained using fructose as source of carbohydrate by keeping the hormonal treatment constant through leaf explants (Talukdar and Ahmed, 2004). It was reported that 30 g L^{-1} fructose concentration resulted with highest growth rate (547%) of fresh callus biomass as compared to maltose (60 g L^{-1}) which showed least growth rate (212%) after 45 days. In contrast to these findings, the results of present investigation were very encouraging since early induction and rapid proliferation of calli could be achieved at comparatively low concentrations of growth hormones. The callus growth was rated as 'very good' (++++) in four point scale in three treatments (T11, T12 and T41). In these treatments, fastest callus initiation was recorded within 12-16 days in leaf and within 14 days in nodal explants.

The effect of exposure of light and darkness was also studied simultaneously for *Aquilaria* callus morphology. The results revealed that the callus remained creamish yellow or white in colour in dark conditions (Fig. 2e-h) which on exposure to light (20-40 μE m^{-2}/S^{1}) developed pigmentation and turned green (Fig. 2f). The texture and type of callus was influenced by the type of growth regulators and explants used. Four distinct types of calli were obtained from the explants of *Aquilaria malaccensis* Lam. i. e. white, yellow white to cream, blackish white and yellowish brown callus. Loose and fragile white callus was obtained when MS media supplemented with high concentrations of 2,4-D with low concentrations of Kinetin were used (Fig. 2d-e) and placed in dark. It turned green within 2-3 weeks after transferring to light conditions (Fig. 2f). When

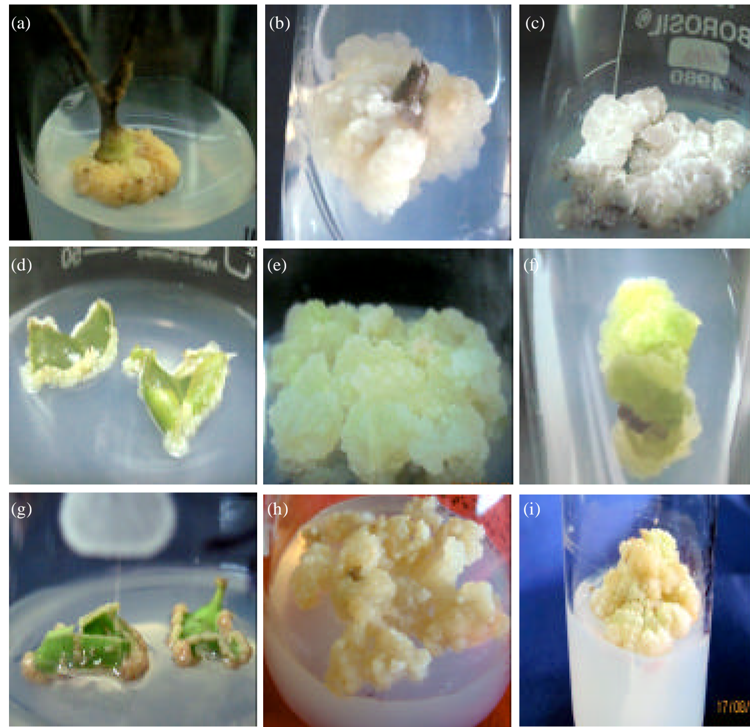

Fig. 2(a-i): Effects of culture media, growth regulators and explants on callus induction and growth: (a-b): Callus induction from nodal explants in Murashige and Skoog (MS) medium, (c): Callus from leaf explants in MS+coconut water, (d-f): Callus growth in MS medium+2,4 Dichlorophenoxyacetic Acid and Kinetin, (g): Callus induction in woody plant medium; h and i: Callus growth in MS+α-Naphthalene Acetic Acid and 6-Benzyladenine purine

supplemented with high concentrations of NAA and less BAP, yellow white to cream colored and compact callus was observed (Fig. 2h-i). MS medium supplemented with CW produced blackish white and compact callus (Fig. 2c); however, the callus obtained on WPM was pale yellow or brown in colour, compact and globular in nature (Fig. 2g). Similar types if callus initiation have been reported *Aquilaria malaccensis* by earlier workers also (Talukdar and Ahmed, 2001). In nodal explants, callus induction was slow and took more time than that of leaf explants (Fig. 2a-c).

Embryogenic and organogenic callus were also noted in various treatments from leaf and nodal explants. Embryogenic callus formed somatic embryos in MS medium with or without growth regulators (Fig. 3c and d). Root formation from callus tissue were achieved in half strength MS medium supplemented with NAA (1 mg L^{-1}) alone (Fig. 3a and b). This is the first report of success of rhizogenic and embryogenic callus obtained in *A. malaccensis* species. Complete organogenesis from *A. malaccensis* callus tissues could not be achieved as also reported earlier (Tiengtum, 1995). Previous studies on growth characteristics of callus tissue of *Aquilaria malaccensis* revealed that callus tissue did not regenerate into whole plant (Talukdar and Ahmed, 2001). However, direct and successful extraction of the agar oil has been achieved from calli of *A. malaccensis* (Talukdar *et al.*, 2002). The important biochemical constituents such as sesquiterpenoids, the major components of agar oil, may be synthesized in living cells using calli and chromone derivatives and

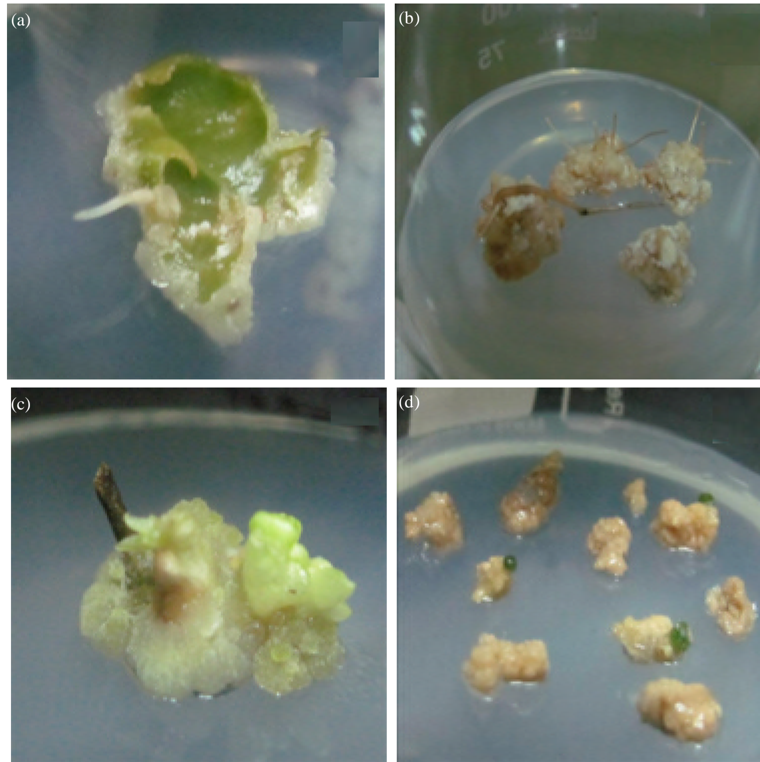

Fig. 3(a-d): Organogenic callus of *Aquilaria malaccensis* Lam. resulting in half strength Murashige and Skoog (MS) medium, (a-b): Root proliferation in MS+α-Naphthalene Acetic Acid (1 mg L^{-1}), (c): Embryo formation in MS+6-Benzyladenine Purine (0.75)+IAA (0.3 mg L^{-1}) and (d): Embryo formation in basal MS medium without any hormone

may be produced from debris of dying callus cells (Okudera and Ito, 2009). Hence such large scale and rapid callus production protocol could be employed for *in-vitro* biosynthesis of such biochemical principles in laboratory conditions. This may help in reducing uncontrolled extraction and over exploitation of this critically endangered tree species for commercial purposes while conserving in the wild natural habitats of region in particular and other habitats in general in the near future.

CONCLUSION

A standard and reproducible tissue culture protocol for rapid induction of callus has been developed using leaf and nodal explants of *Aquilaria malaccensis* Lam. Use of young fresh leaves on MS nutrient medium supplemented with low concentration of auxin and cytokinin was found most suitable for rapid callus initiation in *A. malaccensis* as compared to nodal explant and WPM may serve as alternate source of nutrient medium. The hormonal combination of 2,4-D 2 mg L^{-1}+ Kinetin 0.1 mg L^{-1} in MS medium is recommended to obtain good quality callus mass with high production percentage. Rapid multiplication and maintenance of calli were observed in MS medium supplemented with CW. Four morphologically different types of callus were observed as influenced by different hormonal combinations and light conditions. Rhizogenic and embryogenic callus were also derived. It was concluded that use of juvenile leaf explants (approx. 3-6 months old) and MS

medium supplemented with 2, 4-D and kinetin is most suitable and reproducible protocol for induction of viable and good quality callus of *Aquilaria malaccensis* Lam. The same could be utilized for organogenesis and direct agar oil extraction from this critically endangered but commercially important tree species.

ACKNOWLEDGMENT

The authors are thankful to the All India Council Technical Education, Government of India, New Delhi for financial assistance provided under their RPS programme. Sincere thanks are also due to the Director, NERIST for providing administrative and lab facilities in carrying out this research work.

REFERENCES

Barden, A., N.A. Anak, T. Mulliken and M. Song, 2000. Heart of the Matter: Agarwood use and Trade and CITES Implementation for *Aquilaria malaccensis*. Traffic International, Cambridge, ISBN-13: 9781858501772,.

Bhuiyan, N.I., J. Begum and N.H. Bhuiyan, 2009. Analysis of essential oil eagle wood tree (*Aquilaria agallocha* Roxb.) by gas chromatography mass spectrometry. Bangladesh J. Pharmacol., 4: 24-28.

Burkill, I.H., 1966. A Dictionary of the Economic Product of the Malay Peninsula. Ministry of Agrinutrient, Kuala Lumpur, Malaysia, pp: 198-206.

Chakrabarty, K., A. Kumar and V. Menon, 1994. Trade in Agarwood. WWF-TRAFFIC, India. India 51.

George, E.F., 1993. Plant Propagation by Tissue Culture: Part 1, The Technology. 2nd Edn., Exegetics, Edington, UK., pp: 337-356.

Gupta, M., V. Mohankumar and K.K. Janardhanan, 1995. Distribution of Vesicular arbuscular mycorrhizae in some important medicinal and aromatic plants. KAVAKA, 22/23: 29-33.

Kim, Y.C., E.H. Lee, Y.M. Lee, H.K. Kim, B.K. Song and E.J. Lee, 1997. Effect of aqueous extract of *Aquilaria agallocha* stems on the immediate hypersensitivity reactions. J. Ethnopharmacol., 58: 31-38.

Kosmiatin, M., A. Husni and I. Mariska, 2005. Germination and propagation agarwood in *in vitro*. J. Agron. Biogen, 1: 62-67.

Kumari J.P., M.P. Asokan, S. Sobha, L. Sankari Ammal, K. Rekha, R.G. Kala, R. Jaysree and A. Thulaseedharan, 1999. Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell.). Argic. Curr. Sci., 76: 1242-1245.

Litz, R.E., P.A. Moon and M.C. Victor, 1995. Somatic embryogenesis from leaf callus derived from mature trees of cycard *Ceratozamia hildae* (Gymnospermae). Plant Cell Tissue Organ Nutrient, 40: 25-31.

Lloyd, G. and B. McCown, 1981. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by shoot tip culture. Int. Plant Prop. Soc. Proc., 30: 421-427.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Najar, Z.A., S. Agnihotri and A.K. Awasthi, 2012. Conservation of biodiversity of some important medicinal plants of tropical forests of India through tissue nutrient. Int. J. Sci. Nature, 3: 233-236.

Okudera, Y. and M. Ito, 2009. Production of agarwood fragrant constituents in *Aquilaria* calli and cell suspension nutrients. Plant Biotechnol., 26: 307-315.

Okugawa, H., R. Ueda, K. Matsumoto, K. Kawanishi and A. Kato, 1996. Effects of jinkoh-eremol and agarospirol from agarwood on the central nervous systems in mice. Planta Med., 62: 2-6.

Omar, M.S. and F.J. Novak, 1990. *In-vitro* plant regeneration and Ethyl Methane Sulphonate (EMS) uptake in somatic embryos of date palm (*Phonenix dactylifera*). Plant Cell Tissue Organ Nutrient, 20: 185-190.

Palit, M.K., 1996. *Aquilaria agallocha* Roxb: A commercial agar-oil yielding tree of the north east India. Arunachal Forest News, 14: 30-33.

Rai, M.K., 2010. Review: Biotechnological strategies for conservation of rare and endangered medicinal plants. Biodiversitas, 11: 157-166.

Rao, R. and K. Vaidyanath, 1997. Callus induction and morphogenesis in sesame (*Sesamum indicum*). Adv. Plant Sci., 10: 21-26.

Saikia, M., K. Shrivastava and S.S. Singh, 2012. An efficient protocol for callus induction in *Aquilaria malaccensis* Lam. using leaf explants at varied concentrations of sucrose. Int. J. Plant Res., 2: 188-194.

Saikia, P. and M.L. Khan, 2013. Population structure and regeneration status of *Aquilaria malaccensis* Lam. in homegardens of Upper Assam, northeast India. Trop. Ecol., 54: 1-13.

Talukdar, A. and G.U. Ahmed, 2001. *In vitro* induction and growth characteristics of callus of *Aquilaria agallocha* Roxb. From north-eastern region of India. Asian J. Microbial. Biotech. Env. Sci., 3: 53-57.

Talukdar, A. and G.U. Ahmed, 2004. Effect of sugars on the growth of callus in *Aquilaria agallocha* Roxb. (Thymeliaceae). Proc. National Acad. Sci. India Section B: Biol. Sci., 74: 85-90.

Talukdar, A., G.U. Ahmed and S.K. Dutta, 2002. *In vitro* produced agaroil from callus nutrient of *Aquilaria agallocha* Roxb and analysis of its chemical constituents by GC/MS. Koryo, Terupen oyobi Seiyu Kagakunikansuru Toronkai Koen Yoshishu., 46: 350-350.

Tiengtum, P., 1995. *In vitro* culture of agarwood trees (*Aquilaria* spp.). Master Thesis, Graduate School of Kasetsart University, Bangkok (Thailand).

Verma, P., A.K. Mathur, S.P. Jain and A. Mathur, 2012. *In vitro* conservation of twenty-three overexploited medicinal plants belonging to the Indian Sub Continent. Sci. World J., 10.11.