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Direct somatic embryogenesis in *Myristica malabarica* Lam., an endemic, threatened medicinal species of Southern India and detection of phytochemicals of potential medicinal value

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Abstract: Direct somatic embryogenesis was obtained from intact and fragmented zygotic embryos of *Myristica malabarica*, an endemic, threatened medicinal species of Western Ghats of Southern India while cultured in Murashige and Skoog medium containing activated charcoal. In the absence of activated charcoal there was no embryogenic response but only callus formation in zygotic embryos and their fragments. The addition of gibberellic acid resulted in the emergence of shoot from the somatic embryos. The various developmental stages of somatic embryos were examined using scanning electron microscope. Thin layer chromatography revealed the presence of compounds similar to lignans in the embryogenic mass. GC-MS analysis of the embryogenic mass revealed the presence of several compounds of potential clinical value including malabaricone A , α-spinasterol and γ- sitosterol. The spent medium showed strong anti-bacterial activity against *Pseudomonas aeruginosa*. The results are significant since this is the first report of tissue culture and induction of somatic embryogenesis in *Myristica malabarica.* The embryogenic culture system is potentially useful for efficient and consistent production of bioactive compounds and also has strong implications for conservation of its valuable germplasm.

Keywords: *Myristica malabarica, activated charcoal, somatic embryogenesis, lignan.*

Abbreviations: BA: 6-benzylaminopurine, 2,4-D: 2,4-dichlorophenoxyacetic acid, 2-iP: 2-isopentenyl adenine, GA_3: gibberellic acid, GC-MS: gas chromatography-mass spectrometry, HDMS: hexamethyldisilazane, HPLC: high performance liquid chromatography, IAA: indole-3-acetic acid, IBA: indole-3-butyric acid, MS: Murashige and Skoog, NAA: naphthaleneacetic acid, SEM: Scanning electron microscopy, TDZ: thidiazuron, TLC: thin layer chromatography.

Introduction

Myristica malabarica Lam., an important medicinal plant commonly known as Malabar nutmeg, rampatri or Bombay mace, is a rare and threatened endemic species (Varghese & Krishnamoorthy, 2006; Ravikumar & Ved, 2000) of Western Ghats of South India. It belongs to an archaic group of flowering plants, the Myristicaceae. It is a medium- sized tree with single-seeded fruits used in traditional medicine for the treatment of indolent ulcers and rheumatism. It is among the highly traded top twenty medicinal plants in India (Ved & Mudappa,1999) and one of the major ingredients of several Ayurdevic (Indian medicine) preparations- Baladir taila and Muthu Marunthu. Muthu Marunthu is reported to possess anti-tumour effect (Palani *et al.,* 1999). There are recent reports of its anti-oxidant activity (Maity *et al.,* 2007), superoxide scavenging and cytotoxic properties (Khanom *et al*., 2000; Pham *et al*., 2000) as well as anti-ulcer effect (Maity *et al.,* 2007) and nematicidal activity (Choi *et al.,* 2008) and several classes of therapeutically active compounds including the acylphenols - malabaricones (Patro *et al.* 2005; Bauri *et al*. 2006) and isoflavones including biochanin (Talukder *et al*., 2000) have been isolated from it . The species is red listed (IUCN, 2008) and is threatened due to extensive damage resulting from unregulated lopping for its fruit , large scale and indiscriminate collection of the wild material (Mathachen *et al*., 2004; Daniels *et al*., 1995) and habitat shrinkage. The seeds are vulnerable to temperature and humidity, while the fruit perishes very soon. Regeneration by conventional methods is hampered by the recalcitrant nature of seeds (Anil Kumar *et al*., 2002), a long growth period and scarcity of propagules and is inadequate to meet the demands of the herbal and pharmaceutical industry. Owing to the enormous medicinal potential of this species it is essential to apply *in vitro* approaches to augment the availability of plant material to facilitate a steady supply of biomass for production of its valuable medicinal compounds and to aid the conservation of its valuable germplasm for maintenance of biodiversity and sustainability of the ecosystem. However culture of woody species is difficult and work with tropical woody angiosperms including fruit and nut trees (Gavinlertvatana,1992; Gomez & Litz, 2002; Merkle & Nairn, 2005) has lagged far behind herbaceous species mainly because of limited availability of explants, polyphenolic exudation and poor germination (Jain, 2006) and there is no report of *in vitro* culture of this species. The present study was undertaken to investigate the morphogenetic potential of zygotic embryos of this species and the phytochemical profile of the cultured tissues and the spent medium and their biological activity. Induction of direct somatic embryogenesis from zygotic embryos, detection of lignans and identification of compounds with medicinal potential including malabaricone A, spinasterol and γ-sitosterol in the embryogenic cultures and the detection of antibacterial activity in the spent medium are being reported.

Materials and methods

Plant Material and culture conditions

Freshly harvested, ripe fruits of *Myristica malabarica* were obtained from Tropical Botanical Garden and Research Institute, Trivandrum, Kerala and Panja Range Forest, Puttur, Dakshina Kannada, Mangalore. The fruits (9 cm in length, 5 cm in diameter) were washed thoroughly with liquid detergent and surface-sterilized

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using 0.1 % mercuric chloride ($HgCl_2$) for 10 min. These were then given four to five rinses with sterilized distilled water in a laminar flow hood and then slit open to expose the seeds surrounded by the yellowish aril. The intact and broken zygotic embryos of medium (0.7-0.9 cm in length) and large (1-1.3 cm in length) size were cultured in MS medium (Murashige & Skoog, 1962) with different combinations of the growth regulators – IAA, NAA, 2,4-D, BA, 2-iP, TDZ, kinetin, zeatin and with or without 0.25 % activated charcoal (Table 1). The pH of the medium was adjusted to 5.6- 5.8 prior to addition of 0.8% agar followed by sterilization in an autoclave at 121° C and 1.1 kg cm^{-2}. All cultures were incubated at 25 ± 2°C in continuous light with a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1} provided by 40W cool white, fluorescent tubes. Sub-cultures were carried out at four to six week intervals.

The data were subjected to one way analysis of variance (ANOVA) and the difference between the means was compared by Fisher's least significant difference test (LSD). Percentage data were arcsine square root transformed prior to analysis.

Histological analysis: was carried out according to Johansen (1940).

Scanning electron microscopy

Somatic embryos were fixed overnight in 2.5 % glutaraldehyde in 0.1 M Hepes buffer, pH 7.2 (with 0.02% Triton X -100). The fixed material was washed thrice with buffer followed by dehydration in a graded ethanol series - (25, 50, 70, 85 and 95% ethanol) each for 10 minutes and 20 minutes in 100% ethanol. The dehydrated samples were then processed by chemical drying as follows: 2 parts 100 % ethyl alcohol / 1 part HDMS (hexadimethylsilazane), 1 part of 100% ethyl alcohol / 2 parts of HDMS for ten min each then 2 changes for 15 min each with 100 % HDMS. The samples were then air-dried in a hood overnight after removing HDMS, mounted on metal stubs and splutter coated with gold and palladium for 3 min and observed in a scanning electron microscope (FEI Quanta 200).

Enviromental scanning electron microscopy

Specimens were viewed fresh with a FEI Quanta 200 environmental scanning electron microscope.

Phytochemical profiling of the embryogenic cultures and spent medium of M. malabarica

Extraction of phytochemicals from the embryogenic mass: Lignans and other phenolic compounds were isolated from the embryogenic mass as described by Koulman *et al*. (2001). The embryogenic mass was crushed in liquid nitrogen followed by extraction of the powder with 80% methanol. This was followed by further extraction with dichloromethane and an equal amount of water and centrifuged for 6 min at 1000 g. The aqueous layer was discarded and the organic layer was analysed by TLC and GC-MS.

Thin layer chromatography of the dichloromethane extract of the embryogenic mass: TLC of the dichloromethane extract was carried out on silica gel 60 (Merck) plates developed with chloroform: methanol (9: 1) or toluene: ethyl acetate (9.8: 0.2). The compounds were detected by spraying with methanol: sulphuric acid (1:1) and heating at 150°C for 2 min or visualized in an iodine chamber.

GC-MS analysis of the dichloromethane extract of the embryogenic mass: GC-MS analysis of the dichloromethane extract using a Perkin Elmer Clarus 500 GC- MS system with an Elite5MS column (30 m × 0.25 m, film thickness 0.25 µm). The operating conditions were as follows: Helium carrier gas flow: 1mL/min; split ratio 5:1;Temperature programming: initial temperature,150°C, ramp of 15°C per min to 320°C, hold for 5 min , Injector temperature : 260ºC ; E.I.: 70 eV; ion source temperature: 200° C; mass scan range of 34-600 amu. The Turbomass software with NIST GCMS library was used to analyse the eluted volatiles and to identify each compound.

Extraction of phytochemicals from the spent medium

GC-MS analysis of the ethanolic extract of the spent medium: The spent medium extracted with 80 % ethanol was evaporated *in vacuo* for GC-MS (Agilent 5973-6890) analysis using a DB-5 capillary column (30m x 0.25m : film thickness 0.25 µm). The operating conditions were as follows: Helium carrier gas flow: 5 mL / min; Temperature programming: 70ºC (3 min), ramp of 10°C per min to 280°C, hold for 10 min, Injector temperature: 250ºC; E.I.: 70 eV; mass scan range of 30-450 amu. The ChemStation software with Wiley GC MS library was used to analyse the eluted volatiles and identify each compound.

Assay of anti bacterial activity of extract of spent medium of M. malabarica by disc diffusion method

The anti- bacterial activity of the ethanolic extract of the spent medium against selected gram-positive and gram-negative organisms was assayed by the disc diffusion (Jorgensen *et al*.,1999) method using 5-10 µL of extract adsorbed on the surface of sterile Whatman No.1 filter paper disks (6 mm in diameter). All tests were performed in duplicate. Commercial anti-bacterial test discs containing ciprofloxacin (5 µg / disc) were used as positive controls.

Results and discussion

Responses of zygotic embryos of medium size and induction of direct somatic embryogenesis in media with activated charcoal

There was massive leaching of phenolics from the zygotic embryo explants into the medium in 1-2 days after culture. The nature of the response elicited depended upon the presence and absence of activated charcoal in the medium irrespective of the hormonal combinations employed. Explants of intact or broken zygotic embryos of medium size cultured in media without activated charcoal turned green and underwent callusing (Fig. 1a). Direct induction of somatic embryos was obtained in intact or

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Fig.1. Effect of activated charcoal on in vitro responses of cultured zygotic embryos of medium size. (a) Callusing in media without activated charcoal and with 26.9 µM NAA ,4.40 µM BA , 0.45 µM TDZ; (b,c &d) Direct somatic embryogenesis in media with 0.25% activated charcoal - (b, c) Formation of green somatic embryos at varying developmental stages in media with 8.8 µM BA and with activated charcoal; (d) Bipolar somatic embryo in media with 14.8 µM 2-iP and 1.35 µM TDZ and activated charcoal . (bar = 1 mm)

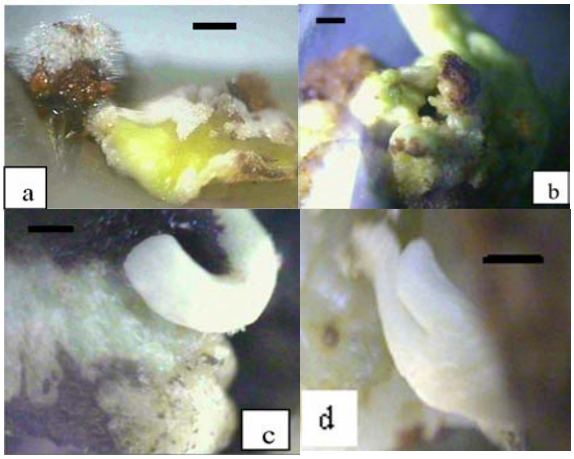

Fig. 2. Electron microscopic studies of somatic embryogenesis from zygotic embryos of medium size cultured in media with activated charcoal . (a) E- SEM micrographs showing proembryonic globules ; (b, c) Scanning electron micrographs showing somatic embryos singly and in clusters ; (d) Scanning electron micrographs showing direct development of cotyledonary somatic embryos from zygotic embryos.

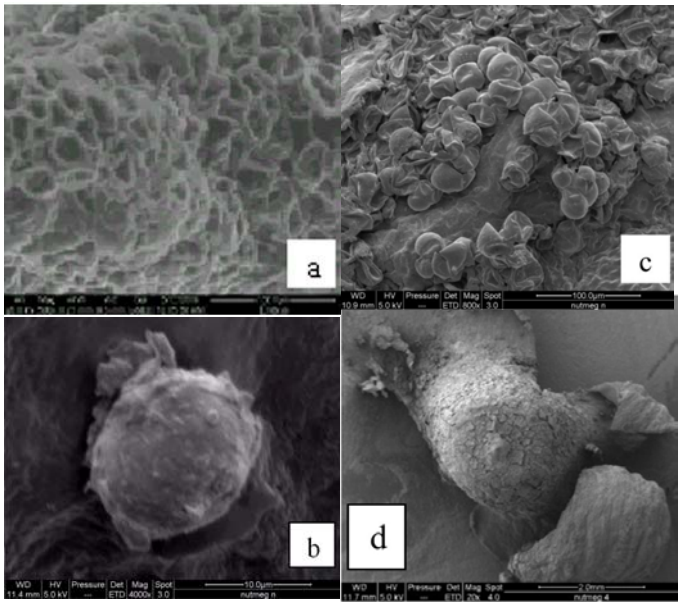

Fig. 3. Histological studies of somatic embryogenesis (a) Embryogenic cells; (b,c) Heart-shaped and (b) cotyledonary forms (bar =1 mm)

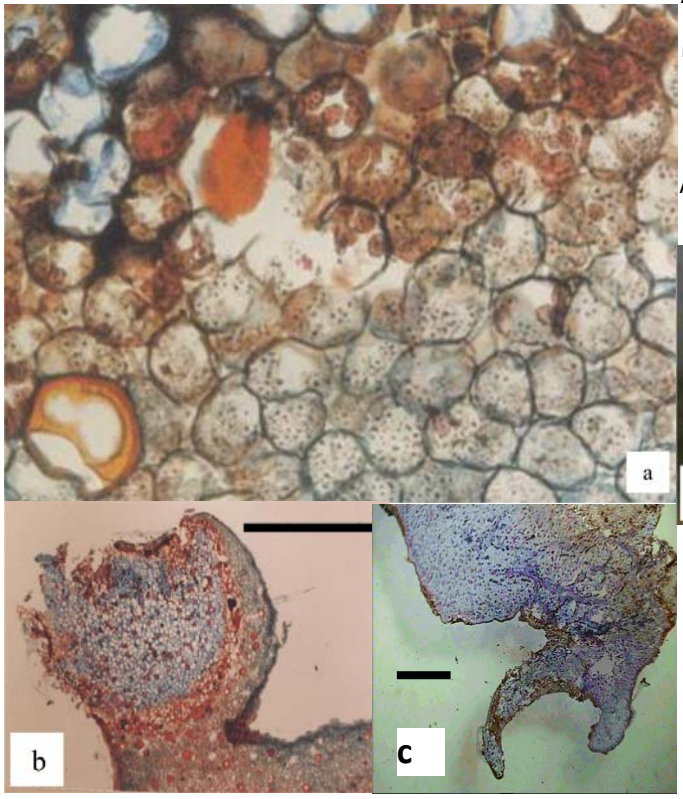

Fig. 4. Development of somatic embryos of diverse forms in different media with activated charcoal. (a) Leafy (in media with 23.2 µM kinetin and 5 µM NAA); (b) Multicotyledonary (in media with 22.5 µM BA, 0.45 µM TDZ); (c) Tubular (in media with 22.84 µM IAA,3.55 µM BA ,0.45 µM TDZ) ; (d) Fused and atypical forms (in media with 5.37 µM NAA and 8.90 µM BA); (e) Secondary somatic embryos proliferating massively on transfer to media with 0.89 µM BA and reduced concentrations of activated charcoal (0.05 %) (bar =1mm)

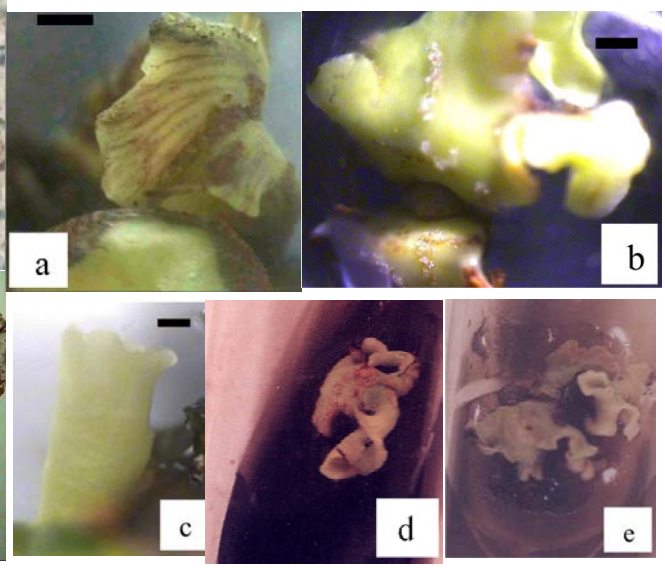

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Table 1.Responses of zygotic embryos of M. malabarica in MS media with activated charcoal and with different combinations of growth regulators (µM)

Sl.No.	BA	2,4-D	IAA	Kinetin	GA_3	NAA	TDZ	2-iP	% response ◊
1*	-	9.0	5.7	-	-	-	-	-	67.87(56.40)e
2**	-	11.36	14.25	11.6	-	-	-	-	100(90)a
3	-	4.5	5.7	-	-	-	-	-	83.3(72.34)bc
4	-	9.0	-	-	-	5.4	-	-	74.95(60.28)de
5	4.40	-	-	-	-	26.9	0.45	-	94.44(80.36)ab
6	22.20	-	-	-	-	-	0.45	-	100(90)a
7	22.20	-	-	-	14.43	-	0.45	-	100(90)a
8	22.20	-	-	-	14.43	-	0.45	-	75(67.5)cd
9	-	-	-	-	-	0.43	0.18	19.7	100(90)a
10	22.20	-	-	-	-	-	0.45	-	100(90)a
11***	-	-	-	-	-	26.85	0.45	-	100(90)a
12	-	-	-	-	-	4.3	0.18	19.7	75(67.5)cd
13	11.10	-	-	-	-	26.85	0.45	-	83.3(72.34)bc
14	-	-	-	-	-	26.85	2.5	-	75(67.5)cd

**15 % coconut milk added; **10 % potato extract added- 100g potatoes boiled, mashed, cooled and filtered; ***50 mg L^{-1} glutamine added; ◊ Values in parentheses are arcsine transformation values of percentage of response.Means followed by the same letters are not significantly different at the P<0.05 level according to Fisher's LSD test.*

broken zygotic embryos of medium size after one week to ten days without any intervening callus phase in different media formulations all with activated charcoal regardless of the hormonal combinations used (Table 1). In media without activated charcoal no embryogenic response was observed and only callusing was obtained (Table 2).This indicated that activated charcoal is obligatory for the embryogenic response. The somatic embryos turned green in two weeks after culture. Several media formulations were tested and it was observed that all formulations with activated charcoal regardless of the hormonal combinations were highly effective in induction of somatic embryogenesis. Stereo-microscopic (Fig. 1b, c and d), E-SEM (Fig. 2a) and electron microscopic (Fig. 2 b, c) studies revealed the presence of somatic embryos at varying developmental stages. Scanning electron microscopy revealed direct development of somatic embryos (Fig.2d). Anatomical studies revealed the presence of embryogenic cells in the cultured tissues (Fig 3a). Early heart stage somatic embryo with distinct protoderm and vascular strand arising from the epidermis of the explant (Fig. 3b) and cotyledonary forms (Fig.3c) were also observed. By sequential subculture in charcoal media with same or different combinations of growth regulators the embryogenically competent state could be maintained over a period of 10 -12 months resulting in establishment of long term cultures. An *in vitro* protocol for the continuous production of the valuable biomass was obtained.

Table 2. Responses of zygotic embryos of M. malabarica in MS media with different combinations of growth regulators (µM) and without activated charcoal

BA	NAA	TDZ	% response ◊
22.20	-	0.45	87.5(75)a
4.40	26.9	0.45	87.5(75)a

◊ Values in parentheses are arcsine transformation values of percentage of response. Means followed by the same letters are not significantly different at the P<0.05 level according to Fisher's LSD test

Table 3. Some of the compounds detected by GCMS of the dichloromethane extract.

Peak No.	*Compound*	*Retention time (min)*	*Area (%)*
11	*Malabaricone-A*	*11.31*	*8.46*
13	*α-Spinasterol*	*12.72*	*0.46*
14	*γ-Sitosterol*	*13.11*	*1.44*

Somatic embryos of variable morphologies ranging from leafy (Fig. 4a), multicotyledonary (Fig. 4b) to tubular (Fig. 4c) forms were observed in various MS media formulations with activated charcoal (0.25%). Fused and atypical (Fig. 4d) forms were also observed. The observations are consistent with the reported occurrence of abnormal somatic embryos in many woody plants (Canhoto *et al.,* 2006) . On transfer to media with reduced levels of activated charcoal (0.05%), massive proliferation (Fig. 4e) of the embryogenic mass by secondary somatic embryogenesis was observed.

Activated charcoal was essential for maintenance of embryogenic response since subculture in media formulations without activated charcoal resulted in callusing (Fig. 5a, 5b) and loss of embryogenic response. Development of red pigmentation was observed in the callus (Fig. 5b).

Germination of somatic embryos by shoot emergence and elongation was observed on transfer of the embryogenic mass to media with GA_3 (Fig. 6). On transfer to media with 18 .7 µM IBA, 10.7 µM NAA and 6 % sucrose formation of root primordia was observed at the base. However the percentage of somatic embryo germination was very low which can be correlated with earlier reports that the presence of abnormal forms (Distabanjong & Geneve,1997; Canhoto *et al.,* 2006) being a major impediment to the commercial application of somatic embryogenesis (Trigiano *et al.,* 1999) in woody species.

Morphogenetic responses of plantlets developed from cultured intact zygotic embryos of large size

Intact zygotic embryos of large size cultured in media with activated charcoal germinated *in vitro* to form plantlets in which formation of adventitious shoots from margins of cotyledons (Fig.7a) was observed . Sprouting

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of axillary buds on the lower nodes of these plantlets was obtained in media with adenine sulfate (Fig.7a , b) indicating their regenerative potential . Media with 15% coconut milk and without activated charcoal could induce callusing in root explants from these plantlets.

Phytochemical analysis of the embryogenic mass

TLC of the dichloromethane fraction of the methanolic extract resulted in the detection of the presence of compounds with the characteristic colour reaction of lignans on spraying with methanol: sulphuric acid (Fig. 8) after development in chloroform : methanol (9 :1). GC-MS analysis of the dichloromethane fraction of the methanolic extract revealed the presence of several peaks. Mass spectrograms of three total ion chromatogram (TIC) peaks (Table 3) revealed the presence of compounds with mass spectral fragmentation patterns similar to the acyl phenol malabaricone - A (Fig. 9a) reported to have anti-leishmanial (Sen *et al*. 2007) , nematcidal (Hosoi *et al*. 1999) effect and cytotoxic activity against human nasopharynx carcinoma KB cell lines (Pham *et al.,* 2000) .Other compounds detected in the dichloromethane extract included α-spinasterol (Fig. 9b) with potential for

Fig.5. (a) Callusing in the embryogenic mass on transfer to non - charcoal media with 22.62 µM 2,4- D and 24.60 µM 2-iP ; (b) Development of red pigmentation in the callus on subculture in 22.62 µM 2,4- D, 5.37 µM NAA, 4.40 µM BA, 0.91µM zeatin, 15 % coconut milk and with no activated charcoal (bar = 1mm)

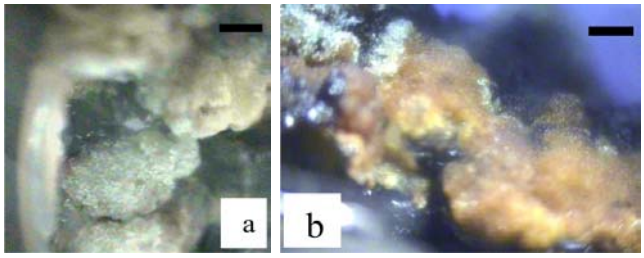

Fig. 6. In vitro plantlet formed by germination of somatic embryos on transfer to media with 14.43 µM GA_3 , 26.9 µM NAA, 4.4 µM BA, 6% sucrose and with activated charcoal

Fig.7. Responses of cultured zygotic embryos of large size. (a,) Formation of plantlets with adventitious shoots on cotyledons; (b) Sprouting of axillary buds on nodes of the plantlets obtained in media with 22.2 µM BA , 5.4 µM NAA , 100 mg L^{-1} adenine sulfate and activated charcoal . (bar=1mm) .

Fig. 8. Mass spectrogram of three total ion chromatogram (TIC) peaks similar to (a) Malabaricone-A, ; (b) α-Spinasterol ; (c) γ - Sitosterol .

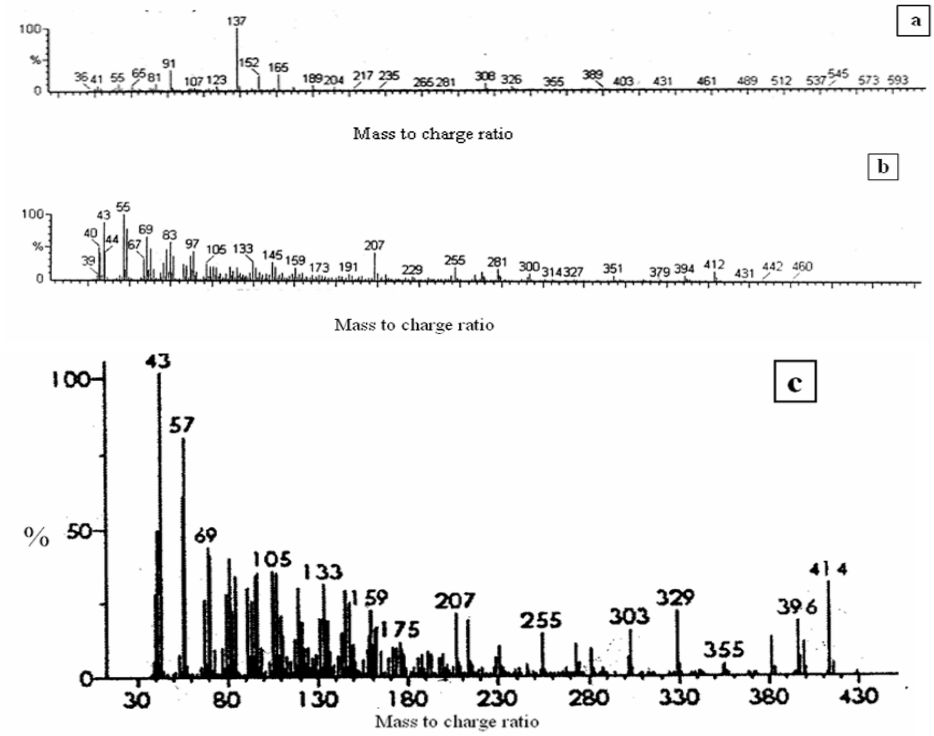

Fig. 9. TLC of dichloromethane fraction of the embryogenic mass of Myristica malabarica

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control of diabetic nephropathy (Jeong *et al.,* 2004) and γ-sitosterol (Fig. 9c) with cytotoxic properties (Khan & Mlungwana, 1999).

Antibacterial activity

Among the four organisms tested, strong anti-bacterial activity of the ethanolic extract of the spent medium against *Pseudomonas aeruginosa* was observed (Table 4) presumably due to the compounds leached out by the explants into the spent medium and this is significant since it indicates the possibility for the development of new anti-infective agents from the spent medium.

Table 4. Anti-bacterial activity (disc diffusion assay) of the compounds leached out in the spent medium

Organism	Volume of the extract (µL)	Diameter of the inhibition zone(mm)
P. aeruginosa	*5*	*19b*
	10	*40a*
S. aureus ATCC 25923	*5*	-
	10	-
S. typhi ATCC 6539	*5*	-
	10	-
E. coli ATCC 25922	*5*	-
	10	-

Means followed by different letters are significantly different at the P<0.05 level

Detection of the active principle in the spent medium in M. malabarica by GC-MS analysis: GC-MS analysis of the ethanolic extract of the spent medium (Fig. 10) revealed the presence of multiple peaks among which a single peak was found to be predominant. The presence of strong anti - bacterial activity in the spent medium can be correlated with the well-recognised role of the synergistic interactions of multiple components of phytomedicines at single or multiple target sites in potentiation of their therapeutic effect (Briskin, 2000).

The findings of the present work assume importance since to the best of our knowledge this is the first report of direct somatic embryogenesis in *Myristica malabarica*.

To conclude, the potential of the embryogenic mass for production of metabolites of medicinal value especially malabaricone-A reported to have anti-cancer properties and anti-leishmanial effects from the cultured tissues has been revealed. Other phenolics including lignans, sterols including α-spinasterol and γ -sitosterol have also been detected in the embryogenic mass. Highly effective media with activated charcoal for initiation of embryogenic tissue has been developed and the role of activated charcoal in maintenance of the embryogenic response has been demonstrated. Furthermore the spent medium of the cultures has also been shown to have strong anti-microbial activity against *Pseudomonas aeruginosa.* The results indicate that this is a good system for production of the medicinal compounds of *M. malabarica* since the *in vitro* produced biomass and the spent medium may be utilized as chemically consistent bioresources for year-round production and possible large-scale extraction of the phytopharmaceuticals from this valuable genetic resource after further refinements in technology to improve the yield. Since the seeds of this rare, slow-growing tree are recalcitrant, the embryogenic mass can serve as renewable and readily available source of plant material with potential applications to relieve the pressures of commercial exploitation by wild harvesting and consequent biodiversity loss. It is also ideally suitable for utilization in strategies for *ex-situ* conservation by cryopreservation (Panis & Lambardi, 2006) and can facilitate exchange of germplasm. Though low rates of germination of somatic embryos is a major limiting factor in the micropropagation of woody species (Jain, 2006) it is significant that germination of somatic embryos by shoot emergence has been achieved in media with GA_3 and the technique can be further refined for *in vitro* rooting and establishment under *ex-vitro* conditions. The data obtained in this study can form the basis for further investigations of the biosynthetic potential of the embryogenic mass and spent medium for the possible application in the development of lead molecules for use in new therapeutic strategies.

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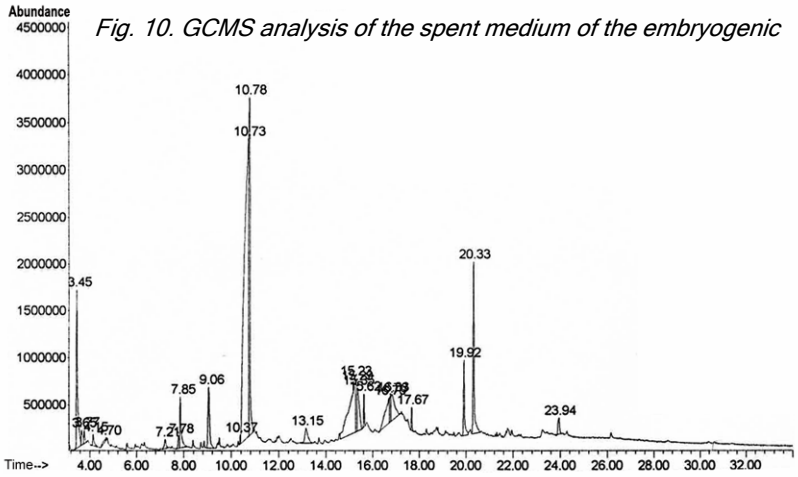

Fig. 10. GCMS analysis of the spent medium of the embryogenic

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